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Shivendu Ranjan · Nandita Dasgupta
Eric Lichtfouse *Editors*

Nanotoxicology and Nanoecotoxicology Vol. 1

Environmental Chemistry for a Sustainable World

Volume 59

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Nanotoxicology and Nanoecotoxicology Vol. 1

 Springer

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ISSN 2213-7114 ISSN 2213-7122 (electronic)
Environmental Chemistry for a Sustainable World
ISBN 978-3-030-63240-3 ISBN 978-3-030-63241-0 (eBook)
<https://doi.org/10.1007/978-3-030-63241-0>

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The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Nanotechnology has a wide range of applications, making it one of the most futuristic technology. This volume includes the basic concepts and principles of nanotoxicity and nanoecotoxicity in general as well as in-depth knowledge on the toxic effect of nanomaterials on microorganisms and plants. Asmatulu has elaborated in Chap. 1 the basic information on nanotoxicity. All the principles and basic concepts are discussed in detail. Suleyman Tekmen discusses in Chap. 2 the effect of nanomaterials on health. In Chap. 3, Cattaneo thoroughly reviews the effect of nanomaterials on reproduction and embryo development. Chhipa in Chap. 4 discusses nanotoxicity in general to microorganism for various potential applications. Karahil in Chap. 5 reviews the cytotoxic and genotoxic aspects of nanomaterials. Khare discusses in detail nanotoxicity as positive response to control growth of drug-resistant bacteria in Chap. 6. In Chap. 7, Correa et al. address toxicity response of man-made nanomaterials to organisms living in water bodies. Various in vitro methods used for the in vitro toxicity assessment of drug delivery-based nanocarriers are discussed in Chap. 8 by Souto et al. In Chap. 9, Ebrahimnejad et al. discuss the impact of nanomaterials on the food chain. Kumar et al. review the effect of nanoparticles on the growth and development of various plants in Chap. 10. Chapter 11 gives a concluding insight into the field of nanotoxicology and nanoecotoxicology.

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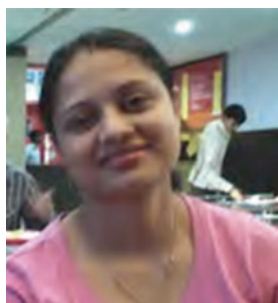
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Chapter 1

Nanotoxicity and Nanoecotoxicity: Introduction, Principles, and Concepts



Shawn Hughes and Eylem Asmatulu

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Abstract Nanotechnology is the creation, processing, characterization, and application of materials at nanoscale, which is in the range of one-billionth of a meter. It can also be associated with systems or processes that offer goods as well as services at this scale. Nanotechnology research and development has been expanding rapidly, and this trend will likely increase worldwide for the next couple of decades. Innovation in nanotechnology may result in business fluctuations, such as rapid obsolescence of existing technologies and capital moves from traditional business to novel technology. Nanotechnology offers many benefits to many industries, including medicine, energy, manufacturing, aircraft, defense, and food, although it has its own risks. Thus, engineers, scientists, and policymakers should work cooperatively to produce safer nanomaterials for community use and minimize the adverse effects of nanotechnology and its products. Here, we review the effect of nanomaterials on the environment, health, economy, and society. We also evaluate the growing market share of nanotechnology products in the United States and other

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V. Kumar et al. (eds.), *Nanotoxicology and Nanoecotoxicology Vol. 1*,

Environmental Chemistry for a Sustainable World 59,

https://doi.org/10.1007/978-3-030-63241-0_1

nations. Finally, we summarize the preferable nanomaterials for different applications, their health effects, and safe handling techniques.

Keywords Nanotechnology · Market · Toxicity · Environmental Health and Safety

1.1 Introduction

Throughout history, scientists have made vast improvements in technology and nanotechnology without concern for the environmental, health, social, and economic impacts of the research. Over the past decade, there has been rising concern about the impacts of nanotoxicology and nanoecotoxicology, which represent innovative and promising research areas in the biological field of toxicology. Nanotoxicology has been defined as the study of the toxicity and impact resulting from the production of nanomaterials (IUPAC 2007). Nanoecotoxicology has been defined as the scientific study of the impending danger to living organisms and ecosystems resulting from the harmful consequences of exposure to nanoparticles; the mechanisms of their action; and the diagnosis, prevention, and treatment of nanoparticle intoxication (Buzea et al. 2007; Asmatulu et al. 2014). These new research fields deal with the assessment and procedures involving the toxicological properties of nanoparticles, with the purpose of influentially discovering the degree of environmental or biological threat they pose. Researchers further characterize the properties of nanoparticles by their size, shape, surface area, surface charge, crystal structure, coating, as well as solubility/dissolution. The most important factors in their consideration are the environmental factors of temperature, pH, ionic strength, salinity, and organic matter, which together influence the development and properties of nanoparticle behavior and eventually their toxicity (Walters et al. 2016). Nanomaterials have been defined by the scientific community as materials with an external dimension in the nanoscale distribution size of 1–100 nanometers (nm) (10^{-9} m), and with an internal arrangement or surface structure measured in the nanoscale range (Buzea et al. 2007; ISO 2008). Nanomaterials are considered to be natural occurring, incidental, or manufactured. They are comprised of 50% or more of particles at nanometer size and distributed in an unbound state, such as an aggregate or agglomerate (IUPAC 2007).

Naturally occurring nanomaterials are made as secondary by-products of volcanic ash, soot from fires, or combustion processes. These by-products are normally physically and chemically heterogeneous and can be defined as ultrafine particles. Researchers and manufacturing companies have always engineered nanomaterials with a specific design and purpose in mind, which has been accomplished through their physiochemical properties, material characterization, and function (Safenano 2018). The ideology of nanotechnology cannot be invented by one individual. However, Richard Feynman hypothesized the idea of manipulating particles, atoms, and molecules in 1959 which was proven by Professor Norio Taniguchi in 1974. These new methodologies of nanomaterials have evolved into a vast variety of

research and development in almost all industries. Even though the philosophy of research into nanotechnology has been found for the past 60 years, the impact of these new nanomaterials, nanoscale processes, and nanotechnology-based manufacturing entities has not been completely understood. Research in nanotechnology has allowed scientists to begin manipulating the molecular design of a material's substructure and environmentally developed nanoparticles, which has changed the properties of materials to benefit the given industry. But the adverse chemical, environmental, or synthetic design of nanoparticles could cause defects that are more harmful than good. Because of the depth of research that has gone into nanotechnology, scientists must also understand the impact that these new materials will cause. This has led to new methodology and research, including the possibility that nanotechnology will cause adverse reactions and not just positive outcomes. This new ideology has created a new methodology for nanotechnology waste and modification standards. The new methodology has become important to researchers, environmentalists, and society. The focus of this chapter is on introducing the classifications, current state, market impact, and safety issues that have risen in the recent years.

1.2 Study of Nanotoxicity and Nanoecotoxicology

Ecotoxicology has been described as the study of the effects of toxic chemicals on biological organisms, populations, communities, and ecosystems (NOAA 2017). Ecotoxicology is a field that incorporates toxicology and ecological methodology. The ideology of this slant is to predict the effects of toxins on organisms. This prediction helps discover the most efficient and effective action to prevent or remedy any harmful effect on biological entities, and to identify and classify the harmful effects of the toxin. As knowledge about nanotechnology increases, it has become necessary to evaluate the effects of nanomaterials on environmental organisms. This has led to the emergence of nanoecotoxicology, a new branch of ecotoxicology. Here, the specific goal has been devoted to engineered nanomaterials. The evaluation and study of environmental hazards of nanomaterials is needed because a harmful experience will likely occur during the lifecycle of a nanoproduct. The ability for researchers and scientists to perform a valid risk assessment will be an important step on the road to a justifiable development of nanotechnology and comprehensive acceptance in the society. Review boards to develop standardized test methods and standardized operating procedures, similar to those of the Environmental Protection Agency, to identify adverse effects of nanomaterials are still being developed and discussed across all scientific fields.

An understanding of how nanomaterials network with other organisms on a molecular or cellular level is needed to create testing and experimental procedures. Research is limited on the different types of nanomaterial molecular interaction with organisms. Table 1.1 lists the nanoparticles and associated research studies conducted to date.

Table 1.1 Nanoparticles and associated research studies

Nanomaterials	Toxicity study on	References
Titanium dioxide	Aquatic (algae – <i>Raphidocelis subcapitata</i>), antimicrobial, and cellular (<i>Escherichia coli</i>) organisms	Ozkaleli and Erdem (2018), Ranjan and Ramalingam (2016)
Iron oxide	Toxicity on rainbow trout, zebra fish	Zhu et al. (2012), Ozgur et al. (2018)
Gold nanoparticles	<i>Drosophila melanogaster</i> , mouse fibroblasts	Coradeghini et al. (2013)
Carbon fullerenes	Aquatic (embryonic zebrafish, benthic organisms) and bacteria	Oberdörster et al. (2006)
Nanosilver	Aquatic organisms (Japanese rice fish – Medaka, <i>Oryzias latipes</i> , <i>Eurasian perch</i> , <i>Danio rerio</i> , fish red blood cells), roundworm (<i>Caenorhabditis elegans</i> , benthic organisms), rabbits, broiler chickens, and mammalian cells in vitro	Kaphle et al. (2018), Pulit-Prociak (2015)
Silicon dioxide	Mouse fibroblasts and lung epithelial cells	Okoturo-Evans et al. (2013)
Zinc oxide	Mammalian cells in vitro, mouse fibroblasts, microalgae (<i>Scenedesmus obliquus</i> and <i>Chlorella vulgaris</i>), bacteria, and earthworms	Baysal et al. (2018), Bhuvaneshwari et al. (2015), Zhang et al. (2016), Senapati and Kumar (2018)
Copper oxide	Mammalian cells in vitro (kidney cells, lung epithelial cells), green algae, plant (Indian mustard- <i>Brassica juncea</i> L.)	Perreault et al. (2012), Da Costa and Sharma (2016), Nair and Chung (2015)
Quantum dots	In vivo toxicity of mice and rats, aquatic	Libralato et al. (2017)
Cerium oxide	Plants (rice and potato), algae, in vitro cell, rats	Taylor et al. (2016), Forest et al. (2017), Khorrami et al. (2019)
Aluminum oxide	Plants (<i>Arabidopsis thaliana</i> , <i>Allium cepa</i>), algae, mice, fly (<i>Drosophila melanogaster</i>)	Rajeshwari et al. (2015), Dev et al. (2018), Shirazi et al. (2015)

Results from these studies showed that soluble nanoparticles had the possibility to have toxicity to all organisms. Many research studied proved that the adverse effects usually come from ions released from cobalt, silver, and some other nanoparticles. To examine the toxic properties of nanomaterials, one needs to investigate where toxicity is found (mainly research laboratories and industry) and which ion interacts with. This includes the investigation of the absorption of nanoparticles by living cells, soils and organic material, and the impact of nanoparticle deposition in the environment. Nanomaterial interactions and impacts must be understood before scientists and the U.S. Food and Drug Administration approve the use of these materials.

Nanomaterial toxicity can be classified based on the immense research and studies of nanotoxicants. They can further be identified as a hazard by means of ecotoxicological data, as well as through their physical–chemical properties, biodegradability, accumulation, particle size, and composition. The four main sources of nanoparticle release whereby the ecosystem is contaminated are

intentional, nonpoint source, accidental, and point source. All four methods by which nanoparticles are inadvertently released result in them being absorbed by the environment and humans, either through the soil where agricultural crops are grown or through groundwater contamination. Intentional release is a designed method of deliberately releasing nanoparticles into the ecosystem for the purpose of biodegradation and absorption by the environment. Here, nanoparticles are released into water or soil, causing them to interact or diffuse with other molecules, which is referred to as nanoparticle aggregate diffusion. The contaminated molecules then seep into groundwater, which is inadvertently consumed by animal and plant species as well as humans in the area. Aggregation kinetics is essential in the disposal methodology of nanoparticle aggregates and can be studied by the nanoparticle mass (N) and fractal dimensions d_f using molecular dynamic simulation software, such as LAMMPS, Desmond, GROMOS, or Materials Studio, in the presence of solvent molecules. Through simulation, the diffusion coefficient (D_o) for the aggregates can be determined to scale $D_o \propto N^{-df}$ (Fraaije et al. 2018).

Nonpoint source release occurs when contaminants are released into the environment over an extensive area. These are some examples (Huang et al. 2008):

Air: When a car is running, the engine produces a variety of chemical products, including oxides of nitrogen (some of which are toxic) and molecules of unburned hydrocarbons from gasoline. Similarly, in industrial areas, pollutants and soot are caused from combustion processes such as burning.

Water: As the result of nanomaterials being released into the environment, acid rain can form, changing the pH balance of the water, which can enter the water cycle. The result can be catastrophic to the ecosystem, harming the fish populous and other creatures that depend on freshwater lakes and streams.

Soil: Hazardous nanomaterials can enter the soil from the air or water, thereby changing the soil due to the absorption of the nanomaterial. Occasionally, these nanoparticles are absorbed by plants, which in turn causes the plants to become toxic.

The accidental release of nanomaterials occurs during production or in factories by means of human error or through the waste stream, although environmental regulations have been put in place to ultimately limit this type of release.

Point source release of nanomaterials has been defined by the U.S. Environmental Protection Agency as any single detectable source from which pollutants are released, such as a ditch, pipe, ship, or factory flue gas stack (NOAA 2017). From knowledge of the nanomaterial being released in this manner, the U.S. Environmental Protection Agency can regulate the amount that is released and can try to hold companies responsible for this waste. However, unregulated release of nanomaterials from point sources can have considerable consequences, resulting in water pollution and unsafe drinking water, as well as restricting activities such as fishing, water sports, and swimming in these areas. Some of the chemicals released by point sources have been identified as harmless, but others have been noticed to be highly toxic to the ecosystem, humans, and wildlife. Whether the release of a chemical is harmful to the ecosystem in surrounding areas depends on numerous factors such as

type of chemical, chemical concentration, timing of chemical release, weather conditions, and organisms living in the area.

This has been one of the driving forces for nanoecotoxicology and research on the effects of nanomaterial waste and the National Pollutant Discharge Elimination System, which was established by the Clean Water Act. This system was put into place to regulate factories and other point sources by requiring them to obtain a permit before releasing nanoparticles into a body of water.

1.3 Current State of Nanotoxicology and Nanoecotoxicology

1.3.1 Economic

Nanotechnology has expanded rapidly over the last couple of decades in order to meet public demand. This has resulted in the application of nanomaterials in many human's daily activities in different industries such as agriculture, medicine, business, as well as public health. Between 1997 and 2005, the National Science Foundation, private and government sectors, and universities have invested funds in nanotechnology research and development. Government investments everywhere in the world climbed from \$432 million to \$4.1 billion, and conforming industry investment surpassed that of the government by the year 2005 (Ray et al. 2009). Nanotechnology has become a growing industry, which is predicted to achieve a net worth of more than \$125 billion by the year 2024, and products incorporating nanotechnology will continue to contribute to global economy (Research and Markets 2018). There could be upward of two million individuals that will be employed in nanotechnology businesses, and possibly three times that many in supporting jobs. While many projected advantages are connected to this new industrial revolution, there are ever-increasing worries concerning the potential adverse health effects that the introduction of these products to the human anatomy might pose. While not fully understood or confirmed by regulations, research, and new developments, these concerns are well founded, due to the overabundance of research and knowledge connected to the effects of environmental air pollution on human health compared to the nanoparticle component contained within that pollution. Although this has been a lucrative industry, a major question is: will the good from nanomaterials outweigh the harm from nanomaterials manipulated at a molecular level that could harm the environment or human beings? Look at the adverse effects that have been linked to genetically modified organisms, which is essentially genetically engineered agriculture digested by humans but does not have to be identified to the populous. Economically, genetically modified organisms have helped with the shortage of food supplies, but they have also been linked to food allergies and 22 diseases:

Several animal studies indicate serious health risks associated with genetically modified food consumption including infertility, immune dysregulation, accelerated aging, dysregu-

lation of genes associated with cholesterol synthesis, insulin regulation, cell signaling, and protein formation, and changes in the liver, kidney, spleen and gastrointestinal system. (The Thom Hartmann Program 2016)

The following are a list of genetically modified foods (The Thom Hartmann Program 2016): corn, canola, soybeans, cottonseed, sugar beets, Hawaiian papaya, zucchini, yellow squash, sugar derived from genetically modified sugar beets, dairy, and unless labeled “no artificial hormones, recombinant bovine growth hormone, or recombinant bovine somatotropin.”

Recently, the U.S. Regulation of Genetically Modified Crops have been divided among three regulatory agencies, although they all have a different stance, and their genetically modified organisms perspectives are different: U.S. Environmental Protection Agency, U.S. Food and Drug Administration, and U.S. Department of Agriculture. Genetically modified crops have some human welfare applications (Weil 2005; Guleria et al. 2017). Thus, like genetically modified organisms, nanomaterials also have advantages and disadvantages. Nanotoxicity and nanoecotoxicity evaluation helps in selecting the correct form of nanomaterials for safe human and environmental use.

1.3.2 Environmental

Exposure of the population and ecosystems to nanomaterials has increased drastically over the past couple of decades due to the demand for better, faster, and smaller technology. Although nanomaterials offer an obvious benefit, many questions regarding their impact on humans and the environment have not been answered. One of the crucial questions that is being addressed along with a plethora of research has been the toxicity and impact of nanomaterials on the environment, although research is still lacking because of the considerable amount of debate over different methodologies. Researchers must understand the properties and biodegradation characteristics of nanomaterials, which will permit a better understanding of the cellular, atomic, and molecular impact of each material. The accumulation of these materials within cells could modify or manipulate cells in an adverse way, which is why an ideology of the nanotoxicology must be known so that the undesirable properties can be avoided before nanomaterials are used. Before nanomaterials are produced, researchers and large companies need to ask vital questions: What is the toxicity of the material? What are the impacts if used? Are non-nano counterparts less toxic? Is there an alternative. Manufactured nanoparticles exhibit physicochemical characteristics that have exceptional electrical, thermal, and mechanical properties, which make them extremely attractive for applications in the commercial, medical, and environmental sectors (National Research Council 2002; U.S. Environmental Protection Agency 2003; Masciangioli and Zhang 2003; Dreher 2004). At present, information defining the relative health and environmental risk assessment of manufactured nanoparticles or nanomaterials has been very limited

due to the lack of research. Only lately important questions concerning the potential human health and environmental impact of manufactured nanoparticles or nanomaterials have been raised (Dagani 2003; Colvin 2003; National Science Foundation and U.S. Environmental Protection Agency 2003).

A recent workshop co-sponsored by the National Science Foundation and the U.S. Environmental Protection Agency detected a number of critical risk assessment problems regarding manufactured nanoparticles such as exposure to manufactured nanoparticles, toxicology of manufactured nanoparticles, ability to draw conclusion on manufactured nanoparticle toxicity using current particle and fiber toxicological databases, environmental and biological destiny, transport, persistence, and transformation of manufactured nanoparticles, and recyclability and overall sustainability of manufactured nanomaterials.

Over the last couple of centuries, the earth's ecosystem has been threatened by humans and their greed for advancement. If large corporations are allowed to continue using genetically modified organisms and other toxic nanomaterials, extreme adverse reactions to the way of life as we know it will likely result (Kumar et al. 2018).

1.3.3 Social

As discussed previously, advancements in nanotechnology are primarily the result of society's need for better technology. Whether the technology is for faster phones, computers, imaging devices, agriculture, or health-related advances, the methodology and ideology of nanotoxicity and nanoecotoxicity must be researched and understood. Although nanomaterials are socially accepted because of personal needs, as a society, we must hold corporations, governments, and investors accountable for the environmental, health, and social impacts to which nanomaterials are linked. The research and development of better regulations and knowledge will be crucial in the discovery of adverse reactions caused to the human race and ecosystem from the use, modification, and manipulation of nanomaterials. Genetically modified organisms are finally being studied, and it has been proven that they cause 22 diseases, more of which could be found with further research.

1.4 Prospects of Market Impact

Nanotechnology has been extended to commercial use after two decades of both dedicated basic and applied research (Vance et al. 2015). According to the Woodrow Wilson Center's Project on Emerging Nanotechnologies Consumer Products Inventory, more than 1800 nanoproducts are on the market (PEN 2018). These consumer nanoproducts have a wide range of applications, including personal care products, medicine, clothing, sporting goods, as well as contributing to faster planes

and cars, powerful computers and satellites, long-lasting batteries, and better micro- and nanochips (Asmatulu et al. 2012). Although many nanoproducts are available on the market, not all of them are recognized publicly. Currently, 32 leading countries around the world, including the USA, Germany, Korea, UK, China, and Japan, contribute to nanoproduct development, manufacturing, and distribution, as shown in Fig. 1.1 (PEN 2018). As can be seen, the top three nanoproduct development and manufacturing countries are the USA, Germany, and Korea, with 756, 319, and 135 nanoproducts, respectively.

Nanotechnology and its products have revolutionized every industry and attracted worldwide attention. The Project on Emerging Nanotechnologies categorized these nanoscale products under eight application areas: automotive, cross-cutting, appliances, electronics and computers, food and beverage, goods for children, health and fitness, and home and garden. Among these, the health care industry is one of the largest fields in which nanotechnology has made real advancement, with its application in the diagnosis and treatment of illnesses such as heart disease and cancer. Thus, it is predicted that the worldwide nanotechnology market will develop at a compound annual growth rate of around 17% between 2017 and 2024, as indicated in Fig. 1.2 (Business Press Release 2018).

Nanomaterials can be incorporated into many products in various ways such as directly into a product and also surface coating. Nanoproducts contain either a single type of particle (silver, zinc oxide, and gold) or a compound (silver and titanium dioxide together), based on the need. Considering the composition of all nanoproducts listed in the Project on Emerging Nanotechnologies' consumer product inventory, 47% of nanoproducts have a single type of nanoparticle, while the rest have more than one nanomaterial component, such as silver and titanium dioxide. According to Vance et al. (2015), 39 different types of nanomaterial components are cataloged in the Project on Emerging Nanotechnologies' consumer product inventory and grouped into five main categories: metal, carbonaceous, silicon, not advertised, and other. Technically, metals and metal oxides include the highest nanomaterial composition group (37% of products) advertised in the inventory. Considering all global production, the amount of titanium dioxide, silver, silicon

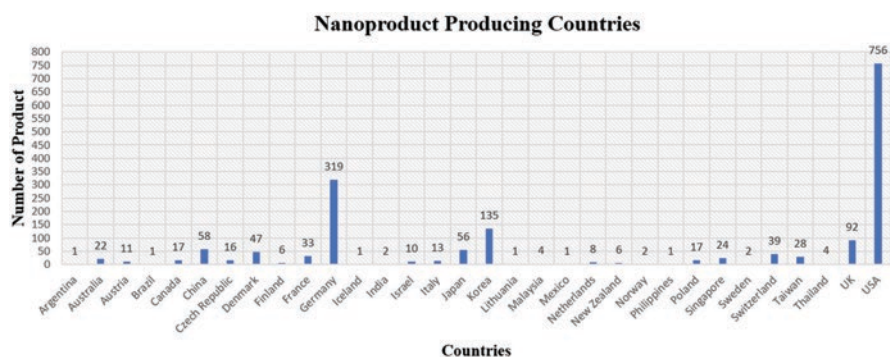


Fig. 1.1 Nanoproduct development and manufacturing countries around the world. (PEN 2018)

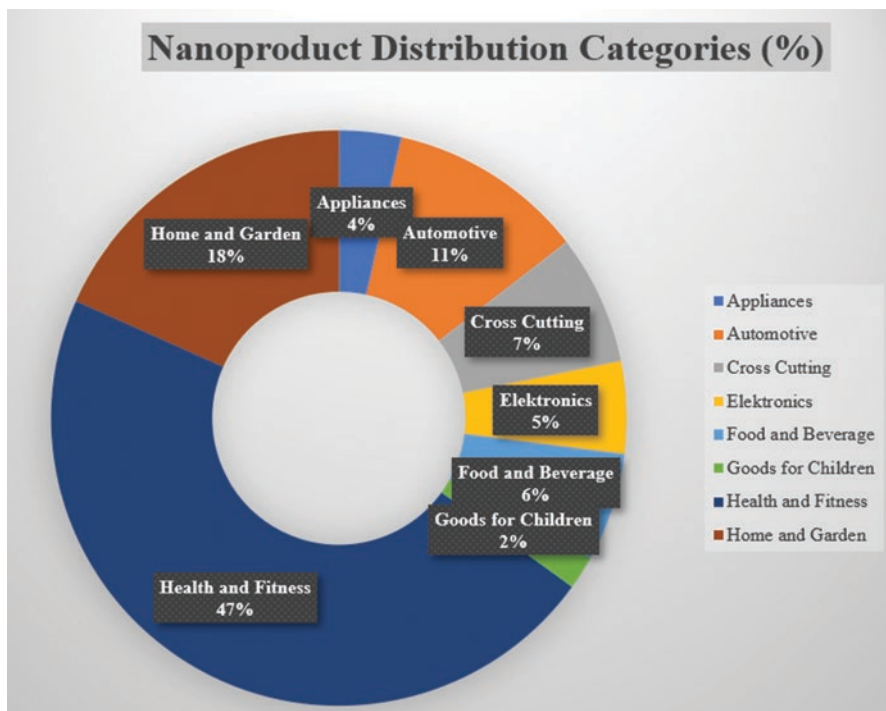


Fig. 1.2 Market share of nanotechnology products based on categories. (PEN 2018)

dioxide, and zinc oxide are the most fabricated nanomaterials. Among these, silver nanoparticles (24 wt%) are the most commonly found nanomaterial in the consumer product inventory. The Project on Emerging Nanotechnologies' consumer product inventory is the largest compilation of nanoproducts available on the market, providing extremely useful information, such as the name of the product, producer country, specific nanomaterial used, product functions, and so on.

However, a full product description is not available, particularly for the mass, volume, and concentration of nanomaterials integrated into products or else the production volume of each product (Vance et al. 2015). Silicon dioxide consumption was 198 kilo tons in 2015 and is expected to reach 768 kilo tons by 2022. Due to its antibacterial property, nanosilver is very popular and has been used in consumer products in a broad range of applications such as electronics, antibacterial textiles, health care, and beauty (Global Market Insights Inc. 2017). The global market growth of silver nanoparticles is expected to be 13% between 2016 and 2024. Additionally, nanocellulose and nanoclay have shown constant growth signs as a result of the increasing scope of their industrial applications. The Transparency Market Research group forecasts that the market revenue of nanoclay and nanocellulose is expected to increase by 24.9% and 19%, respectively, until 2020 (TMR

Table 1.2 Selected nanomaterials market revenue. (Inshakova and Inshakov 2017)

Selected nanomaterials market	Global market revenue by 2016 (USD billion)	Expected global market revenue by 2021 (USD billion)	Expected compound annual growth rate between 2016 and 2021 (%)
Silver nanoparticles	1.1	3	13
Nanoclays	0.7	2.1	24.9
Nanocomposites	1.6	5.3	26.7
Quantum dots	0.61	3.4	41.3
Nanofibers	0.39	2	38.6
Advanced and nanoscale ceramic powders	14.6	22.3	8.9

2015, 2016). Predictions of the selected nanomaterials markets are provided in Table 1.2.

Nonetheless, the Allied Market Research investigators highlight those industries with the most prospects for employing nanomaterials in the production of final merchandise: aviation, automobile, sporting goods, energy storage, electronics, and defense (Allied Market Research 2016). The electronics business has the highest market share (around 30%), while the aviation industry has the quickest developing market because of the expanding use of nanometals, polymer nanocomposites, and corrosion-resistant coatings in the manufacture of aircraft.

1.5 Safety Issues of Nanotechnology Products

Nanotechnology is one of the most significant scientific discoveries of the current times since it offers treatment techniques that none of the previous techniques can deliver. In addition to medical applications, nanotechnology and nanoproducts have a broad range of applications (defense, transportation, and electronics) (Khan and Asmatulu 2013). Thus, the utilization of nanotechnology is very diverse but at the same time dangerous for workers, retailers, and consumers. With new advancements in nanotechnology and nanoproducts, it is important to consider both potential advantages, unintended dangers to human well-being, and conditions that may accompany the improvement and utilization of the innovation. The National Nanotechnology Initiative is focused on the mindful advancement of nanotechnology and its principle targets, such as the environment, health, and safety investigation methodology. This approach incorporates sound logical evaluation of nanotechnology's advantages and risks, and a comprehension of its potential environment, health, and safety effects (NNI 2018). The National Nanotechnology Initiative's environment, health, and safety research strategy incorporates many essential perceptions (risk assessment, product life cycle stages, and exposure) into the basic as well as applied research to identify the possible environmental, health,

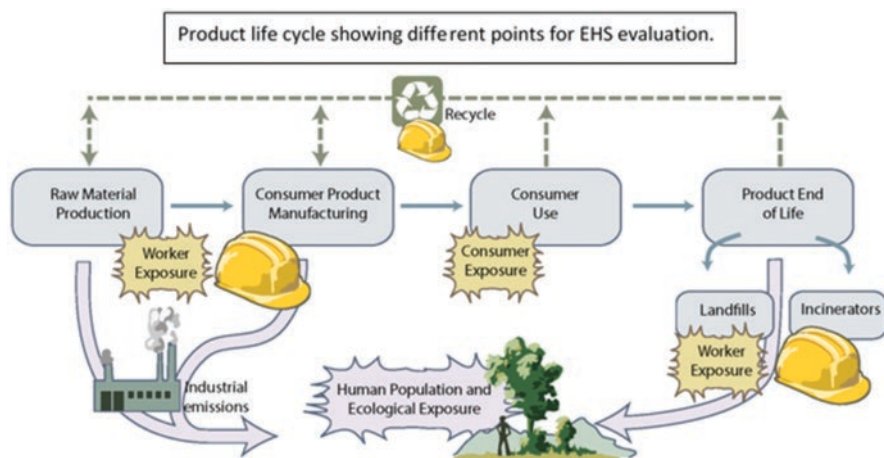


Fig. 1.3 Environment, health, and safety evaluation of nanoproduct life cycle. (Mehta 2014)

and safety effects of nanomaterials. As is shown in Fig. 1.3, environment, health, and safety evaluation of nanoproducts should be investigated by considering the whole life cycle from cradle to grave (NNI 2018).

Risk assessment of nanotechnology is a process of analyzing nanomaterials to provide a better understanding of their possible hazardous effects on humans and the environment. It also links the probability that there could be any exposure to the materials, and level and duration of that exposure (Asmatulu et al. 2017). Despite the risk, if any potential impacts occur, then the individual or the environment must be presented to a specialist for a detailed investigation. Exposure to nanomaterials may occur unexpectedly in the environment or via utilization of nanotechnology-enabled items. The National Nanotechnology Initiative's environment, health, and safety research strategy provides examination studies to create new sets of data in order to address these potential zones of exposure over the life cycle, and it helps organizations to build up their exploration needs and tolerances.

Considering the safety of using nanotechnology and its products, the following are some common concerns for industries and the public. Nanotechnology has many innovative applications whereby it can be advantageous for humanity; however, there are also safety concerns, since nanoproducts are not yet completely tested. One of the safety doubts about nanotechnology in this field includes the utilization of this innovation in therapeutic and health fields, whereby nanomaterials may trigger harmful effects rather than proposed beneficial effects. Since nanotechnology includes the manipulation of matter at the molecular and atomic levels, there are concerns that possible manipulation may result in the creation of materials that will drastically adjust the human lifestyle. This can be particularly seen in the field of biomedical applications where nanoparticles are utilized in different routes, including injectable drugs and sunscreen. Another concern is contamination of the earth's surface, where nanomaterials may build up to toxic contamination levels in

various zones. As such, car engines can discharge gases and emissions to the environment, so car manufacturers in turn create environmental problems since there is a lack of waste-disposal programs.

Nanotechnology is a booming business, providing considerable income to the United States; however, very little effort has been given to environmental and health concerns regarding nanomaterials (Nanogloss 2010).

1.6 Potential Exposure Pathway

According to Vance et al. (2015), the Project on Emerging Nanotechnologies' consumer product inventory has very little information in terms of nanomaterial size and concentration; thus, the real health risks of these products remain virtually unknown. Among all things considered, the consumer product inventory might be valuable for understanding potential introduction pathways from the normal typical utilization of recorded items. In order to examine this utility, a subcategory of 770 products was evaluated to determine the greatest possible routes of exposure of hazardous nanomaterials to humans and consumable products (Fig. 1.4). As can be seen in Fig. 1.4, skin is the primary route of exposure of nanomaterials from the utilization of nanoproducts.

A recent study showed that only 58% of nanoproducts had been analyzed and reported because many products on the consumer product inventory list are solid products that include nanomaterials on the surface and are intended to be contacted, and liquid products that include nanomaterials in suspensions, which are intended to be used on the skin or hair. Among the products analyzed, 25% of them are possibly inhaled during use, such as the case of aerosol sprays and air from hair driers, and 16% of them are ingested by consuming vitamin supplements and throat sprays.

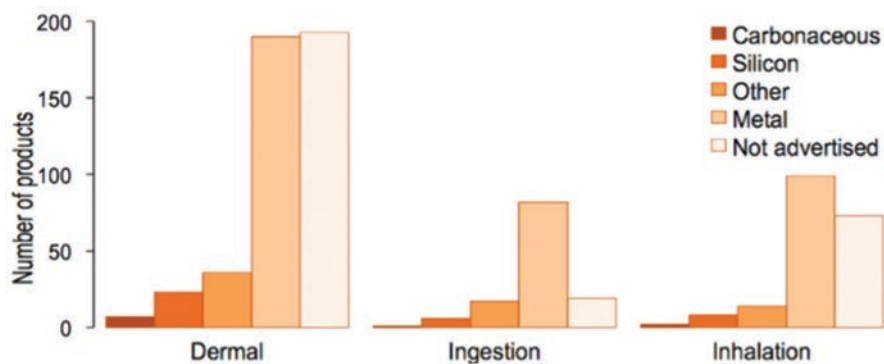


Fig. 1.4 Potential exposure pathways of nanoproducts, grouped by main nanomaterial composition categories. (Vance et al. 2015)

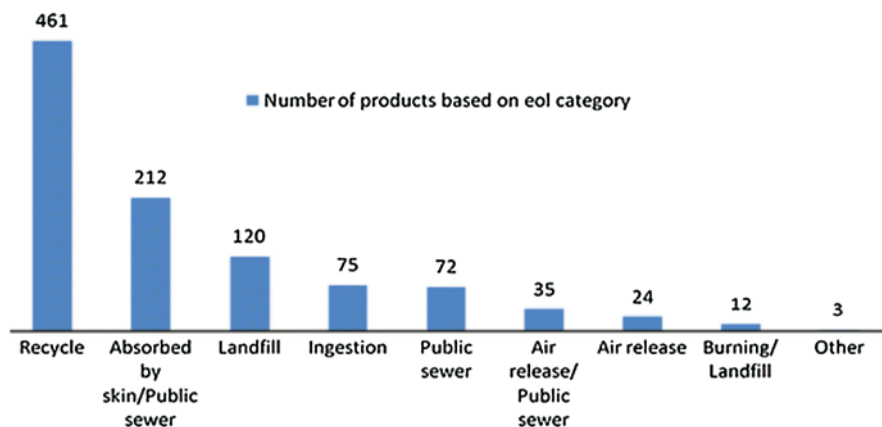


Fig. 1.5 Nanoproduct distribution based on end-of-life category; total Project of Emerging Nanotechnologies consumer product inventory list (1014 products) as of 2010 (Asmatulu et al. 2012)

Asmatulu et al. (2012) analyzed 1014 nanoproducts regarding their end-of-life fate (Fig. 1.5). Results of this study can be used for exposure assessment as well.

The nine end-of-life fate destinations of nanoproducts used by the initial consumer as shown in Fig. 1.5 are discussed above.

Recycling is a procedure of gathering utilized materials to create new items, with the goal that the potential estimation of end-of-life items will not be lost (The League of Women Voters 1993). In this manner, reusing is one end-of-life classification that was utilized.

Absorbed by skin and to some degree washed away and moved to open sewers or a waterway. Nanomaterial-containing products, such as body lotion and sunscreen, are examples of this category.

If there is no feasible alternative, then the landfill is the destination for the end-of-life of nanoproducts.

Ingestion is another fate of nanoproducts taken into the body by drinking or eating. Either these will break up and remain in the body or they will be discharged to the public sewer. The data to isolate maintenance versus discharge were not accessible, so all were recorded as ingested.

Public sewer is another end-of-life category for detergents used for cleaning, which eventually go into the public sewer system. Body creams or sunscreen, which are *absorbed by the skin* and washed away to public sewers or a waterbody, is another category.

Burning/landfilling is another destination fate of nano items (motor oil), whereby nanomaterials can be burned in an incinerator and the ashes sent to a landfill.

Releasing nanoparticles to air is other end-of-life category. For instance, spraying odor-eliminating gases or dissolvable items can be released to air.

Other is the last category where the nanoproduct end-of-life is difficult to classify. For example, fertilizer is given to plants, which can be mixed with surface

water and likely devoured by humans and other creatures. Consequently, it is extremely difficult to characterize a grouping and end-of-life for them.

Genuine proximity in the marketplace is not a huge factor in this investigation, because the products recorded are still in some presence and do exhibit a decent variety of nanoproducts. Bérubé et al. (2010) criticized the first consumer product inventory in 2010, which concentrated mainly on the absence of information relevant to the doses of nanomaterials to which customers may be exposed through consumer product inventory-recorded products (Bérubé et al. 2010). This is a legitimate feedback of the data used to populate the consumer product inventory, which is constructed essentially with respect to promoting claims made by the producers. Nevertheless, the latest adjustments of the consumer product inventory offer a potential solution for information gaps through the commitments of outsider research groups. These alterations are particularly opportune since there is a developing number of distributed investigations that evaluate consumer exposure to nanomaterials released during the utilization of nanotechnology-enhanced products (Royce et al. 2014). Some of these include cosmetic powders, sprays, household products, and products for children (Vance et al. 2015; Nazarenko et al. 2011; Quadros and Marr 2011; Benn et al. 2010; Quadros et al. 2013).

It has been stated that there are no primary standard strategies for surveying consumer risks from utilizing nanotechnology-empowered products or an arrangement of settled upon measurements for portraying nanomaterials to decide environmental focuses (Holden et al. 2014). The advancement of necessary models is greatly needed as a best technique for safe and sustainable nanotechnology improvement in the following decades (Savolainen et al. 2013). Recently, the Consumer Product Safety Commission asked for \$7 million to develop the Center for Consumer Product Applications and Safety Implications of Nanotechnology to create techniques to recognize nanomaterials in products as well as understand human exposure to those materials and toxicity values (CPSC 2016).

1.7 Conclusions

Over the last 60 years, nanotechnology has evolved into a supermarket of research, studies, and products that are available. With the vast amount of resources going into the research and development of new nanotechnology every day, the need has arisen to develop new fields to study the adverse effects that nanoparticle modifications have caused or could cause in the human body and in the ecosystem. Nanotoxicology and nanoecotoxicology have been developed as multidiscipline fields to research the impact of nanotechnology on our own biological ecosystem. Through software and experiments, scientists are starting to unravel the harmful effects of manipulating, manufacturing, and disposing of nanoparticles. This book will present an overall approach on how to test nanomaterials for toxicity, their effects on nanoecotoxicology, nanotoxicology, and summaries of the results that have been discovered in this multidiscipline field to date. Also, an ideological

approach will be shown on the pitfalls and mechanical complications of nanoecotoxicology and nanotoxicology. This has important implications for the test design for nanoecotoxicology and nanotoxicology tests and test conditions, which will lead to educated results.

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Chapter 2

Nanomaterials and Human Health



Süleyman Tekmen and Selda Öksüz

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Abstract In this chapter, nanomaterials are reviewed according to their physico-chemical properties. The reasons for these properties and in turn their behaviors are underlined. Most properties of nanomaterials, such as physical, chemical, electrical, optical, and structural, are strongly size dependent. It has been observed that metallic particles tend to behave like semiconductors, as their dimension decreases. Carbon nanotubes can be engineered to be lighter and stronger than steel. 1D ZnS nanobelts are shown to absorb both UV (320 nm) and visible light (600 nm). Then, how the attributes of nanomaterials can be appropriate for a specific application is discussed. A wide range of nanomaterials and their applications including biosensor, drug carrier, bandage, etc. are concisely addressed. Thereafter, the black side of the nanotechnology, concern related to human health and environment, is elucidated and some experimental studies related to toxicity are compared. It has been shown that their toxicity mechanisms are quiet complex. It has been claimed that generally increase in concentration and decrease in dimensions result in higher toxicity and higher cellular uptake. Copper nanoparticles are shown to be more lethal with

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V. Kumar et al. (eds.), *Nanotoxicology and Nanoecotoxicology Vol. 1*, Environmental Chemistry for a Sustainable World 59, https://doi.org/10.1007/978-3-030-63241-0_2

respect to bulk copper. The toxicity of nanomaterials can be alleviated by coating with suitable materials. Coating CdSe quantum dots with ZnS enhances cell viability by 66%. And finally, the risk assessment and precautions to be taken are given. Some of risk assessment methods are introduced. Due to lack of knowledge, conflicts between results and uncertainty, it is too early to draw a complete picture of nanomaterials in terms of risk assessment.

Keywords Nanomaterials · Applications · Exposure · Toxicity · Protein Corona · Human Health · Risk Assessment

2.1 Introduction: Nanomaterials

Nano word comes from Greek word “dwarf” and, mathematically, it is a prefix defined as one-billionth of a quantity. Generally speaking, nanoparticles are those whose sizes, at least one controllable dimension, are in the range of 1–100 nm. Nanomaterials are developed ones where the nanoscale structure monitored is effectively responsible for the desired behavior of material. Nanotechnology is concerned with the world of invisible particles that are controlled by physical and chemical laws, which are not valid for particles whose dimensions are beyond 100 nm. In other words, it is a field in which both synthesizing and controlling of nanoparticles are achieved at nanoscale or at atomic and at molecular scale. Although there have been many definitions for nanotechnology, one of the most accepted one is the definition by the National Nanotechnology Initiative: Nanotechnology is science, engineering, and technology conducted at the nanoscale (nano.gov).

Nanotechnology provides cutting-edge products that will completely change the way of diagnosis, protection of environment, storage of energy, enhancement of food quality, creating sophisticated structures from single atom devices to macroscale structures. So, in brief, it will become dominant in almost every aspect of our lives in the course of time.

The goal of nanotechnology is to establish control over production of nanomaterials in order to manufacture high-quality, environmentally friendly products which are causing the lowest impact on health and environment. Nanotechnology is a promising technology in solving ongoing problems such as renewable clean energy, clean water, health and longevity, and protection of environment (Nanotechnology and human health: Scientific evidence and risk governance 2013).

Generally, nanomaterials are classified into two groups. First ones are nanoparticles created naturally via a variety of events such as forest fires, volcanoes, dust storms, evaporation of oceans and water. These kinds of nanomaterials coming from natural events are called natural nanomaterials (NNMs). Besides these natural nanomaterials, there are also nanomaterials resulting from human activities such as smoke, exhaust fume, building demolition, cosmetics, and intentionally synthesized

nanomaterials. Nanomaterials of this type, especially intentionally produced ones, are known as Engineered Nanomaterials (ENMs).

Another classification is carried out with regard to dimensions of materials. Zero-dimensional (0D) nanomaterials are materials whose all three dimensions are smaller than 100 nm like nanoparticles quantum dots (QDs). One-dimensional (1D) ones are materials whose only one dimension is bigger than 100 nm, (e.g., nanotubes, nanowires, and nanorods). Two-dimensional (2D) nanomaterials have two dimensions bigger than the upper limit of nanoscale such as nanofilms, nanolayers, and nanocoatings. Three-dimensional (3D) nanomaterials or bulk nanomaterials have no dimensions at nanoscale, but these 3D structures may consist of various nanostructures. All of nanomaterials regardless of their dimension can be metallic, ceramic, or polymeric. Because classification is out of scope of this chapter, we will skip the topic.

In broad sense, two approaches are used in producing nanomaterials. First one is top-down in which starting bulk material is mechanically or chemically reduced to nanoscale material. In this case, a chunk of material of interest is ground or dissolved in an appropriate acid until the desired product is obtained. The second approach is called bottom-up in which nanoparticles are manufactured by chemical processes of atoms or molecules. Because the bottom-up method provides relatively much more control over process, it enables us to carry out the process in a more efficient way and, in turn, reduce pollution.

What makes nanomaterials so appealing is not only their dimensions, but also their intrinsic behavior and functionality due to decrease in size. The behavior of nanomaterials is determined by various parameters, especially size, shape, and quantum confinement effect. For example, metal particles consisting of 50–100 atoms with a diameter between 1 and 2 nm start losing their metallic behavior and tend to become semiconductors (Simon et al. 1993). The parameters affecting behavior of nanomaterials will be explained concisely. It is useful to divide the parameters as physical and chemical parameters.

Physical Parameters

- Size, shape, specific surface area, and aspect ratio
- Aggregation and clustering
- Size distribution of nanoparticles
- Surface morphology
- Crystallinity and defects
- Solubility

Chemical Parameters

- Chemical formula and molecular structure
- Composition
- Phase
- Surface chemistry (surface charge, reactivity, photo-catalytic feature, and zeta potential)
- Hydrophilicity and hydrophobicity

Surface properties like charge and coating, morphology, small size, reactivity, specific surface area, solubility, structure, aspect ratio, production of reactive species, chemical composition and photochemistry are some of the physicochemical properties of nanomaterials. These properties have important effects on toxicity of nanomaterials (Yadav et al. 2014).

Let us try to figure out what happens as size of material decreases. First of all, it is crystal clear that dimensions, at least one dimension, should be shrunk to nanoscale range in order to talk about nanoscale. One of the major roles of particle size and surface area emerges in interactions between nanomaterials and surroundings (e. g., other particles, biological system, and solution). Because specific surface area is defined as surface square meters per gram of material, it is evident that the surface area dramatically increases and results in a more reactive surface determining the way of response of the system. Since the size and surface area are very crucial for interaction, especially in biological system, they should be strictly controlled. For example, a number of studies support the hypothesis that alveolar macrophages are less efficient at engulfing nanoparticles than micron-sized particles (Pratten and Lloyd 1986).

However, it has not been elucidated the relation between shape, aspect ratio and nanomaterial behavior, and studies have shown that these factors have significant effects on nanomaterials toxicity, endocytosis mechanism (uptake of material by cell membrane via either pinocytosis or phagocytosis), and various and complicated chemical interactions. It will be informative to give some behaviors of nanomaterial as determined by the above-mentioned parameters. It has been observed that spherical nanoparticles are easier to be accepted by cell than nanorods and nanofibers (Champion and Mitragotri 2006). For example, uptake of gold nanorods is slower than spherical gold particles (Chithrani et al. 2006). It has also been illustrated that cytotoxicity of nanomaterial is also shape dependent. Hsiao and Huang showed that rod-shaped ZnO particles were more toxic than spherical ZnO nanoparticles in the research in which effects of physicochemical properties on human liver epithelium cell were investigated (Hsiao and Huang 2011). In another experiment, rod-shaped silver nanorods and gold nanorods were manufactured by electron beam physical vapor deposition, and their cytotoxicity on human skin fibroblasts was determined. The findings demonstrated the maximum toxicity in fibroblast cells for both rod-shaped silver nanorods and gold nanorods (Favi et al. 2015). Another factor playing a role in the behavior of nanoparticles is the aspect ratio defined as the ratio of surface area to the volume. It is evident that aspect ratio increases as the size of particle decreases. It has been shown that materials with higher aspect ratio are more likely to be toxic (Shvedova et al. 2005).

The next factor affecting the behavior of nanomaterials is their surface charge. Surface charge, charge per unit area, has significant effects on process, product quality, and performance. In applications related to imaging and drug delivery, surface charge is one of key factors determining the performance of nanocarriers. Surface charge is also responsible for biocompatibility and uptake (Fröhlich 2012). Additionally, it has a significant effect on the clearance of nanoparticles from the immune system (Teodoro et al. 2011). Materials can be positively charged,

negatively charged, or neutral. The sign and magnitude strongly affect the nanomaterial behavior in various media. It has been reported that surface charge and particle composition of nanoparticles are significant factors of their action and toxicity on cellular processes (Beddoes et al. 2015). A positively charged material was shown to penetrate cell membrane more easily than negatively charged or neutral nanoparticles (Georgieva et al. 2011). Also, toxicity of nanomaterials might be determined by surface charge. In a study carried on zebrafish and mice embryos, positively charged PAMAM dendrimers were observed to be toxic, while no toxicity effect was detected for negatively charged PAMAM dendrimers (Heiden et al. 2007). Liu et al. investigated the surface charge effect on cellular uptake, cytotoxicity, and in vivo biodistribution of CdSe/ZnS quantum dots. They have demonstrated that charged particles, especially negatively charged ones, were internalized by macrophage and cancer cells more efficiently than neutral particles. They have also shown that distribution and cytotoxicity of nanoparticles were strongly dependent on surface charge (Liu et al. 2015). Wang et al. have shown that negatively charged Au nanoclusters accumulated in liver and spleen took relatively longer time, while positive Au nanoclusters have an adverse effect on the blood system. They also confirmed that surface charge has a significant effect on tumor uptake. Negatively charged Au nanoclusters exhibited the highest tumor uptake (Wang et al. 2016). Since surface charge has a profound influence on material–biological system interaction, researchers have been trying to modify and control the surface charge of materials in order to overcome their toxicity and benefit from them more efficiently.

Chemical composition medium has also a significant impact on behavior of nanomaterial. It has been demonstrated that the interaction type between the medium and nanoparticles can modify their properties such as size and toxicity. Thus, the same kind of nanoparticles can manifest different toxicity behaviors, depending on the media (Hou et al. 2013). Studies confirmed that chemical composition also influences their toxicity. In a research carried in vivo, it was seen that soluble nanosilver caused toxicity, whereas TiO_2 exhibited no toxic effect (Griffitt et al. 2008).

Like other factors, crystal structure has also a prominent effect on the behavior of material. While some kind of crystal structure “say rutile TiO_2 ” caused DNA damage, other crystal structure “say anatase TiO_2 ” showed no damage to DNA (Gurr et al. 2005a, b).

Agglomeration of nanoparticles is another element responsible for toxicity. Agglomeration is formed by accumulation of particles. Agglomerated particles are generally accumulated in organs like liver, spleen, and lung. Because their excretion from body takes relatively longer time, their adverse effects are also long-term ones. Although they did not exhibit toxicity, it has been shown that agglomerate carbon nanotubes are more harmful than well-dispersed carbon nanotubes (Wick et al. 2007).

The surface morphology of nanomaterials is a very critical parameter controlling their behavior because interaction with media and other particles or molecules occurs via surface. The adsorption of oxygen species, radicals, or transition metals on nanomaterial has a great influence on the physical and chemical properties of nanomaterials and in turn their interaction with other species. It has been shown that

nanoparticles surrounded by reactive oxygen species (ROS) were responsible for inflammation (Li et al. 2010). Because of these undesirable effects, the surface of nanomaterials of this kind is coated and their toxicity is controlled. Connor et al. showed that spherical gold nanoparticles coated with various coatings showed no toxicity response (Connor et al. 2005). Their stability was also enhanced via coating with a suitable material (Kirchner et al. 2005). Scientists have claimed that CdSe quantum dots have substantial cytotoxicity because of their surface oxidation and Cd^{2+} ions releasing. Cd^{2+} ions are carcinogenic to human cells. If the quantum dots are encapsulated with the ZnS, the decrease of the cell viability tended to diminish about 66%. But, encapsulation of quantum dots with 98% bovine serum albumin reduces this rate almost to zero (Derfus et al. 2004). In another study, ZnS and CdSe quantum dots were coated with mercaptoundecanoic acid and sheep serum albumin and their cytotoxicity in vitro was investigated. Scientist exposed human hepatocytes, primate kidney, and cervical cancer cells to these quantum dots and determined a decrease in the cell viability (Shiohara et al. 2004).

Hydrophilicity and hydrophobicity are clear to have a great impact on the interaction of nanomaterial's behavior. Hydrophilicity and hydrophobicity are measures of spreading water on materials. If water spreads across material easily and in turn contact angle is less than 90° , the material is said to be hydrophilic and loving water. If the water molecules show resistance to spreading, then water almost forms a droplet dwelling on the surface of material and thereby the contact angle is bigger than 90° ; the material does not love water and is called hydrophobic material. An interesting phenomenon of nature using nanostructures is the lotus effect (Karthick and Maheshwari 2008). As can be experienced, leaves of the lotus plant are always clean. This is due to hydrophobic property of leaves which protects them against moisture. Each drop of water can remove hydrophilic dust from the leaves. It is crystal clear that when hydrophobic material is in water, these non-polar molecules tend to clump up together in order to achieve minimal contact with water molecules. Thus, nanoparticles should exhibit hydrophilic behavior in order to be well dispersed in water or serum media, while hydrophobicity is required in material–cell interaction. The dispersion of particles can be provided by using hydrophilic functional groups attachment (Fratoddi 2018).

It is well known that solubility is tightly dependent on the interaction between solvent and solute. A strong attraction between them means higher solubility while a weak attraction means lesser solubility. In general rule, like dissolves like. It means that polar solutes tend to dissolve in polar solvent while non-polar one dissolves in non-polar solvent. Another factor influencing solubility is common ion, which decreases the solubility. However, temperature and pressure have a strong impact on solubility; this is out of scope of this chapter. The importance of solubility becomes apparent when considering blood. Because blood mainly consists of water, medicines used should be polar in order to play their roles. Furthermore, their circulation period in bloodstream is longer since their recognition as intruder by immune system is weakened. This is why hydrophobic materials are modified by hydroxylation, amination, or other way. Thus, materials can be used in drug delivery in a more efficient way (Rašović 2017).

2.1.1 *Natural Nanomaterials (NNMs)*

Natural nanoparticles are ones created by various natural events or processes. Only about 10% of aerosol in the atmosphere is generated by human activities while the rest comes from natural activities (Taylor 2002). The main sources of natural particles are volcanic eruption, dust storms from cosmic sources, ocean evaporation, and some organisms. As these processes occur simultaneously and their interactions are extremely complicated, they can end in considerable changes in the atmosphere and in turn living being in it. To give some example, eruption of Krokato in 1883, one of deadly volcanic eruptions, caused a dramatic deviation on the climate and resulted in the global temperature decrease (livescience). Ten cubic kilometer of debris was shot out into atmosphere (Self and Rampino 1981). Gas released during eruptions involves HCl and H₂S, which play a central role in acid rain generally starting just after eruption. This acid rain adversely affects the biosphere.

As mentioned above, this is not the only source. Tunguska fireball in 1908, Russian fireball in 2013, etc. can be exemplified among meteorites, which are another source responsible for production of natural particle (Pichon et al. 2013). According to astronomical observations and analyses carried out on meteorites, stardust mainly consists of carbide, oxide, nitrate, silicate, carbon, and organic-based nanomaterials (Barnard and Guo 2012). Cosmic dust is proven to cause pneumoconiosis and fibrosis in rats when taken thorough intratracheal route (Batsura et al. 1981).

Dust storms are the determining source of nanomaterials in deserts. The earth has many deserts such as Sahara, Arabian, Gobi, Kalahari, and Australian desert. Depending on the power of sand storms, nanomaterials on the deserts can be alleviated and transported to extremely longer distances, say thousands of kilometers. This transportation has also a strong effect on climate stability and biosphere. Dust particles hanging in air, aerosol, are in the range of 100–200 nm by 50% (Shi et al. 2005). Almost in each breadth, we inhale terrestrial airborne dust particle and for now it is impossible to completely stop exposure to them. These particles degrade air quality and lead to some health problems like asthma (Sapkota et al. 2005). When inhaled, metal nanomaterials can reach the lungs and deteriorate the lung function by producing reactive oxygen species (Rašović 2017).

2.1.2 *Engineered Nanomaterials*

Particles produced by natural events sometimes called incidental particles are not controllably created in terms of size, shape, or compositions. In contrast to natural nanomaterials, engineered nanoparticles have been intentionally and precisely synthesized by human to have certain size, shape, functionality, and composition. The earliest engineered nanoparticles dates back to ancient Romans, Egyptians, and Chinese. Craftsmen successfully produced nanoparticles and used them without

knowing the scientific origin behind. They were able to prepare nanoparticle solution of gold and other metals. In ancient times, most of the applications of nanoparticles were mainly related to aesthetics. By combining nanoparticles with glass and jewelry, they achieved many attractive products with magnificent colors. Sometimes, nanoparticles, especially gold nanoparticles, also were used for medical reasons by ingestion of nanoparticles.

As technology advances in synthesis and characterization techniques, many kinds of nanoparticles with varied sizes, shapes, and functionalities have been produced. The main difference from the incidental particles lies in the control over the process of production. By tuning properties of nanomaterial, the application area becomes wider and wider, including new technical materials, batteries for energy storage, sporting goods, catalysts, water purification, soil remediation, cosmetics, medical bandages, car paint and waxes, food packages, toothpastes, healthcare products, diagnosis and therapy, and many others ([understandingnano](#)).

What makes nanoparticles so salient is their unique and tunable features. Because of the rapid technological revolution in the area of nanoparticles, it is not a distant dream to make structures atom by atom. By achieving this, defects adversely affecting attributes of device or structure can be almost totally eliminated and structures with flawless performance can be obtained.

Many features of nanoparticles such as physical, chemical, and electro-optical, and magnetic properties can be tuned via controlling their size, shape, activity, and so on. Since these properties can be modified, thanks to nanotechnology advancement, it is highly likely to produce nanoparticles with a desired quality.

In contrast to bulk materials in which energy band of electrons is continuous, nanomaterials have discrete energy levels due to quantum size effect. Besides these discrete energy bands, another astonishing parameter is mean free path, which is the path taken by charge carries between two subsequent collisions. The number of collisions in bulk material is extremely high, which means relatively short mean free path leading to loss of heat energy while mean free path for nanosystem, collisions and in turn loss as heat energy can be extremely low. Thus, this undesirable heating effect can be compensated by nanostructures and electrical properties on material can be considerably enhanced. The study carried out on nanoparticle-embedded poly-ethylene-matrix by Yurkov et al. revealed that dielectric permittivity and absorption coefficient of matrix increase with increase in the composition ratio of embedded nanoparticle (Yurkov et al. [2007](#)). Another important phenomenon is surface plasmon resonance (SPR). Quite different from bulk materials whose surface electrons have wide range of vibrating frequency, surface electrons (surface plasmon) on the nanomaterials resonate only in certain frequencies. Normally, most of incident photons reflect back when encountering a metallic surface. However, if light wave is in phase with the surface plasmons, resonance phenomena occurs. In this case, most of the light absorbed by plasmons and intensity of reflected light takes a sharp deep. Taking advantage of SPR, many surface plasmon resonance-based analysis systems have been developed in order to analyze biomolecules. They have used glass coated with thin gold. Gold is chosen since it is chemically inert to

most of solutions and solutes. Due to excitation of surface plasmons, nanoparticles can be employed in biomedicine, energy, and environmental protection (Garcia 2011).

The unique properties of nanomaterial make them preferable in a variety of areas like tribology and surface engineering. Mechanical properties of material such as tensile, yield, strength, and toughness are greatly dependent on grain size and their boundaries. Since grain size and boundaries are numerous and their distribution alters through materials, the mechanical performance of conventional materials is adversely affected. It is quite possible to modify both grain size and boundaries, thanks to nanotechnology. Nanotechnology provides us to make the grain size bigger and thereby improve their mechanical behavior. Studies have revealed that the modulus of polystyrene nanospheres, silicon nanoparticles, increases with a decrease in the particle size (Mook et al. 2007).

2.2 Applications of Nanomaterials

Nanomaterials have a variety of applications due to their unique physical, chemical, optical, and mechanical properties. Their applications cover various products including food packaging, sporting goods, apparel industry, car paints and waxes, antibacterial cleansers, cosmetics, fuel catalyst, super capacitors, batteries, electromagnetic interference shielding, solar cells, chemical sensors and biosensors, drug delivery systems, environmental remediation and, so on (azom). In conclusion, nanomaterials are making their ways into all aspects of lives (Chaudhry 2012). Some application areas of nanomaterials are summarized below.

The main reason for using nanomaterials is strongly dependent on the goal to be achieved. While for some applications, surface area per unit mass is more beneficial, and control over chemical and biological activities are preferred for other applications (Chaudhry 2012).

Structures with various properties such as fullerenes, nanodots, nanotubes, graphene, nanoparticles, nanofibers, and nanowire have already been on the market. These materials can offer highly beneficial functionalities. Among this material class, carbon nanotubes (CNT) are promising to replace conventional steel due to their superior mechanical properties. The strength of carbon nanotubes can be as high as 63 GPa, while the tensile strength of steel is about 1.2GPa (Purohit et al. 2014). This provides them to be in the class of strongest materials produced by nanotechnology.

In electronics, there are some reasons for such poor performance and these include contamination, inefficient heat dissipation and bad contacts. Materials with significantly high purity and relatively better thermal conductivity can be produced by nanotechnology. Also, more durable contacts can be formed by convenient nanomaterials. Thus, the performance of electronics is enhanced (scitechdaily). In case of microelectronics industry, miniaturization of device elements is a key challenge. The elements such transistors, capacitors, and resistors can be reduced in size, thanks to nanotechnology and this enables microprocessor to run faster. Also,

nanostructures can be used as optical switch or sensor due to their optical properties. 1D ZnS nanobelts have shown to be sensitive to both 320 nm UV light and 600 nm visible light (Fang et al. 2009a, b). This property provides ZnS nanobelts to be used as light-activated optoelectronic switch.

Nanowires, nanorods, and nanobelts have become common in building high sensitive and high selective sensors owing to their relatively high surface area-to-volume ratios. Some examples of application of these nanomaterials include field-effect transistors, field emitter, diodes, solar cells, generators, gas sensor, biosensors, and photodetectors (Ahmad et al. 2018).

Nanotechnology can be exploited to offer new tastes and flavors. Moreover, it can be used to enhance shelf-life and food hygiene by providing strong and light packaging (Chaudhry 2012).

Carbon nanotubes are relatively strong and flexible. These features help them to be used in sport goods such as tennis rackets, hockey sticks, and fishing rods. Lighter and stronger sporting goods can be produced, thanks to nanotechnology (Aithal 2016).

Another area on which nanotechnology applied is automobile industry. Integrating nanoparticle with automobile paint improves the scratch-resistant property. Because of their reduced size, they are extremely effective in clearing blemishes in paint finishes (Coelho et al. 2012).

Distinctive features of nanomaterials help them to be used in many areas. One of the areas which directly affects the public is textile industry. Nowadays, clothing companies have introduced nanotech fabrics which are non-stain, able to repel liquids, and even more comfortable. Textiles modified by nanoparticles have antibacterial, deodorizing, thermal-regulating and static-free, wear-resistant properties. Anti-bacterial attributes of silver, zinc and zinc oxide make them competitive candidates for new-generation bandages. The special design of bandages with silver nanoparticles enables them to protect the injured tissue against outside microorganisms and facilitates fast healing (Rigo et al. 2013).

Nanotechnology allows us to tune physical, chemical, and mechanical properties of nanoparticles. Thus, nanoparticles of desired attributes such as water-repellent, stain-resistant, corrosion resistant, and self-cleaning can be produced by tailoring their functions. All of these features make them prospective candidates for coating materials. These coatings can be employed on the walls, door, windows, metallic water pipes, cars, and aircrafts and even in inner parts of vehicle like engine. Conventional automobile engines waste significant amount of gasoline due to lack of efficient combustion and cause environmental pollution by emission of carbon, carbon monoxide, and unreacted fuel. Because conventional spark plug electrodes used in burning gasoline are defective and decrease the combustion efficiency, it has become important to replace them with ones that are stronger, harder, and wear-resistant. Because nanomaterials have tunable superior properties, they can be designed to offer solution to the problem. Using additive such as cerium (IV) oxide nanoparticles as catalyst also improves the combustion efficiency (Mei et al. 2016). Another problem is related energy waste due to loss of heat. This problem can be handled by coating engine cylinders with suitable nanocrystal materials which hold

heat more efficiently (Dahotre and Nayak 2005). It is possible to make relatively light structures, especially car body, more resistant to denting and scratching which is very important in terms of durability and fuel consumption.

Resolution of displays is determined by size of pixels. Decreasing pixel size by nanocrystals will provide displays with high definition. Nanocrystalline zinc selenide, zinc sulfide, cadmium sulfide, and lead telluride are potential candidates for improving the resolution of monitors (Dresselhaus 1997).

Another area taking advantage of nanomaterials is structuring tougher and harder tools used in cutting tools. Nano tungsten carbide and titanium carbide are relatively much harder and more wear-resistant compared to their conventional counterparts. These fascinating features enable faster and low-cost production (Fang et al. 2009a, b).

As portable electronic equipment like mobile phone, laptop have increased in number, a great demand for relatively lighter batteries with high energy density has also risen and batteries based on nanomaterials can store more energy than traditional batteries. Both storage capacity and life of the traditional batteries are relatively low. These obstacles can be overcome by replacing them with ones having high storage capacity and life. Using nanocrystals, it is possible to achieve high storage capacitors and batteries. It has been shown that nickel–metal hydride batteries based on nanoparticles require less frequent recharging and last longer due to large surface area, in other words, high storage capacity (Banerjee et al. 2009).

Healthcare and personal care industries are other areas on which nanotechnology has great impacts. Conventional materials used as implant and heart valves wear out quickly and result in frequent and expensive surgeries. Since zirconium oxide ceramics are hard, wear-resistant, corrosion-resistant and biocompatible, they can be an acceptable substitute for traditional implants (Bollen 2017). Another noteworthy nanomaterial is silicon carbide which can serve as heart valve due to its low weight, high strength, wear-resistant and inertness to the biological fluids (Bolz and Schaldach 1990). Typical UV protection lotions do not have the desired quality and their stable duration is low. Nanoparticles of titanium dioxide and manganese (II) oxide have already been used in sun lotions in order to prevent harmful UV radiation (Smijs and Pavel 2011). By reducing free radical in the skin, nanoparticles can prevent premature aging and skin cancer. Similarly, nano-sized iron oxide has been used in lipsticks as pigment (Ali et al. 2016). The applications of nanoparticles are not limited to the already mentioned disciplines. Another tempting application of innovative nanoparticles has emerged in drug delivery systems. Nanotechnology provides effective solutions to the limitations of current medicines, diagnosis and treatment advances in therapeutic and diagnostic applications can open up possibilities to fight and cure cancer and other diseases. Due to their controllable properties, excessive consumption and side effects can be significantly lowered. Medicine of interest can be carried by nanoparticle to the targeted area. Then, therapeutic agents are released in a controlled manner. In this case, the desired amount of suitable nanoparticles is sent to the defected region via direct injection or by using electrical and magnetic fields depending on their properties. When nanoparticles reached the target, they are externally forced to vibrate and generate heat. Thus, controlling both

amplitude and frequency of vibration of nanoparticles, cancerous or morbid parts can be eliminated without causing side effects. Although there are many thermo-responsive systems based on polymers, it has been shown that ceramides can also be used as anti-cancer agent by triggering apoptosis (Stover et al. 2008). Since ceramides are vulnerable to relatively high temperatures, they are usually modified by nanoparticles and they became more stable to relatively high temperatures. Using ceramides coated with hydrophobic nanoparticles facilitates their penetration to the membrane and liberates ceramides by hydrolyzation. It has been proved that absorption properties of nanomaterials can be tuned in order to absorb specific light of certain wavelength. These kinds of drug carrier can be triggered to deliver drug by photo-effect (Bakhtiari et al. 2009). It is clear that it is of vital importance to obtain effects of therapeutic. Magnetic nanoparticles, iron oxide nanoparticles, are extremely useful in evaluation of these effects (Heo et al. 2014).

Concisely speaking, beside controllable precise treatment offered by nanotechnology, its obvious advantage is that it does not require expensive, complicated, and relatively uncontrollable surgeries. Carbon nanotubes which can be in single-walled or multi-walled forms, have great potential in various areas, detection, imaging, and drug delivery. Because of their cylindrical shapes, nanotubes are easily taken by cells and this makes them competitive candidate for intracellular delivery of drugs. Functionalized carbon nanotubes have been already employed in delivery of various drugs, such as methotrexate, paclitaxel, and cisplatin (He et al. 2013). Also, nanotubes can be modified to exhibit multiple behaviors. It has been shown that carboxyl-modified nanotubes can be used for both targeting and killing tumor cells (Tian et al. 2011). Due to their intrinsic optical properties, nanotubes can be used in photothermal therapy. Since nanotubes are shown to have strong absorption in near infrared (NIR), damaged cells or tumor cells in which nanotubes are located can be destroyed by irradiating nanotubes with near infrared light (O'Neal et al. 2004). Nanotubes have high electrical conductivity and electrochemical potential. These intrinsic properties make them suitable for (therapeutic) biosensors. Enzymes attached to nanotubes can be exploited in both detecting glucose level and treating diseases (Zhang et al. 2015). To summarize, nanotube-based nanoproducts have great potential in monitoring, imaging, targeting, and multimodal treatments.

Another issue having profound impact on living being is pollution they are exposed to either directly or via what they eat or drink. Because environment and water have been adversely affected by human activities, these effects should be compensated for healthy life. Does nanotechnology have solution to the problem? The answer is yes. One of the most profound benefits of nanotechnology is desalination and purification of water. There are many promising applications in water remediation. Researches approved that nanoparticles like silicates have ability to adsorb heavy metal and to remove pathogen. They can reduce toxic materials to less harmful species. They have also been introduced in water filter systems and provide purified water (Gehrke et al. 2015).

2.3 Exposure Pathways of Nanomaterials

Knowledge of exposure stages of nanoparticles is of great importance in terms of risk assessment and evaluation. The exposure of nanoparticles can take place via inhalation, ingestion, skin absorption, or injection (Hansen 2012). When human is exposed to nanoparticles, the circulation and their fate vary, depending on the exposure form. In order to comprehend their circulation and fate in the body, these pathways should be elucidated. Below is given a concise way of these pathways.

Inhaled nanoparticles in the air come into contact with body via nasal cavity or oral cavity. After pharynx and larynx, they follow the course of trachea which is covered with tiny hair, cilia. The back-and-forth movement of the cilia protects body against invaders by carrying mucus up and out. Finally, they arrive at the lobes of the lungs via bronchial tubes. The lobes involve small spongy sacs called alveoli where exchange of oxygen and carbon dioxide occurs. The alveolar walls are tiny and composed of epithelial cells and tiny blood vessels called pulmonary capillaries. Thus, all of the parts of respiratory system are likely to be under the threat of exposure. Nanomaterials such as paints and coatings, skin care sprays, sunscreen sprays, food additives and colorings can cause pulmonary inflammation (Viswanath and Kim 2016). Particles with smaller diameter can diffuse more easily into the lung than larger diameter particles (Hoet et al. 2004). de Lorenzo (1970) investigated silver-coated colloidal gold nanoparticles with a size of 50 nm using squirrel monkeys and reported that these nanoparticles can be accumulated in olfactory bulb (de Lorenzo 1970). Accumulation of gold nanoparticles leads to intestinal epithelial cell cytotoxicity. This occurs by mitochondria membrane depolarization and the outcomes give information about the relationship between the size of gold nanoparticles, potential cytotoxicity, and their gastrointestinal uptake (Yao et al. 2015). Another research carried on rats revealed that ^{13}C nanoparticles with a size of 35 nm inhaled were found in olfactory bulb and an increase in the amount of nanoparticles was observed as the exposure duration was prolonged (Oberdörster et al. 2004).

The second route of entry, assimilation via the peripheral nervous system, is achieved by retrograde axonal transport. A number of viruses are known to be able to travel via the trigeminal nerve to the semilunar ganglion in the middle of cranial fossa. Herpes viruses can use both anterograde and retrograde axonal transport via branches of the trigeminal nerve (Chaudhuri and Kennedy 2002). Non-biological nanoparticles have also been observed to undergo retrograde axonal transport from the peripheral nervous system. Hunter and Dey demonstrated the uptake of rhodamine labelled microspheres of 20–200 nm in rats (Hunter and Dey 1998).

There are many reports about nanoparticles inhaled and their side effects. Oberdörster et.al reported that carbon nanoparticles in the range of 20–29 nm was accumulated in the rat liver within 30 minutes' duration of inhalational exposure (Oberdörster et al. 2004). This indicates rapid translocation of nanoparticles (Hansen 2012).

Another pathway via which nanoparticles enter the body is skin absorption. The exposure to nanomaterials from the dermal route is the common way of exposure (Mackevica and Foss 2015). Because skin has the largest area, the probability of exposure is relatively higher and also it is challenging to determine the amount of nanoparticles that crosses skin for the same reason. Therefore, it performs as a primary defense organ in human body and it faces dangerous agents all the time (Hagens et al. 2007). Paints and coatings, skin care (lotion), sunscreen (lotion), air fresheners (spray), and sealants are some of the products to which we expose via dermal routes (Viswanath and Kim 2016). The exposure level can be higher for someone who uses commercial skincare products since a number of commercially available sunscreen products contain titanium dioxide nanoparticles. The presence of titanium in the epidermis and dermis after the use of sunscreen has been reported by Tan et al. (1996). Extreme exposure of titanium dioxide can cause reactive oxygen species production such as hydrogen peroxide, singlet oxygen, free hydroxyl radicals and so may induce oxidative stress, and cause significant damage to DNA (Gurr et al. 2005a, b). In an animal model, Tinkle et al. showed that fine particles of beryllium have the ability to penetrate skin while it was not the case for larger particles (Tinkle et al. 2003). There is a consensus that nanoparticles can be assimilated into the body through skin. A study carried on post-mortem flexed stratum corneum indicated that dextran beads in the range of 0.5–1.0 μm can pass through it. The results obtained by Rouse et al. verified Tinkle findings (Rouse et al. 2007). They demonstrated that buckminsterfullerene amino acid penetrated the skin after mechanical flexing. Lademann et al. revealed that microparticles of titanium dioxide incorporated in sunscreen penetrate the stratum corneum (Lademann et al. 1999). Not only are the particle dimensions significant but also surface charge can be a key factor for penetration ability. Kohlia and Alpar demonstrated that positively charged and neutral latex nanoparticles of 50 and 500 nm could not penetrate the dermis, while negatively charged particles could (Kohli and Alpar 2004). In addition, size and shape of quantum dots were also shown to be decisive in the penetration process (Ryman-Rasmussen et al. 2006). Nohynek et al. reported a detailed study related to skin absorption of nanomaterials (Nohynek et al. 2007). According to the study, there was no significant absorption into the systemic circulation.

There have also arisen questions whether the penetration can be significant for defected skin in some way, sunburn, abrasion, or other mechanisms. Zang and Monteiro-Riviere reported that abrasion of rat skin facilitated quantum dots penetration (Monteiro et al. 2005). However, the effect on skin damaged by other mechanisms (e.g. through sunburn), or on infant skin, remains unknown.

Digestive system is another possible portal for entry of nanoparticles. The digestive system includes gastrointestinal tract, liver, pancreas, and gallbladder. Tract of mouth, esophagus, stomach, small intestine, large intestine, and anus are hollow parts of the gastrointestinal tract. Because nanoparticle production has increased steeply and become widespread almost in every aspect of our life and every parts of environment, the foods we eat and the beverages we drink are extremely likely to contain engineered nanoparticles. Food industry use nanoparticles for various reasons such as increasing shelf life and achieving delicious tastes or unintentionally

involved nanoparticle due to coating, packaging, and filtering. Besides, as the use of nanomaterials increases, the possibility of unintentional ingestion of nanoparticles via food animals, fish, and water also increases (Bergin and Witzmann 2013). Swallowing of mucus secreted by respiratory system can also lead to particle digestion because the mucus is likely to be impacted by nanoparticles. Lomer et al. have estimated that about a thousand particles up to three microns are ingested each day by a person in developed countries (Lomer et al. 2002). These particles generally include titanium dioxide, food colorants and silica. Now let us look at the findings related to exposure of nanoparticles by the digestive system. The absorption of nanoparticles in the gastro-intestinal tract depends on their charge, size, surface chemistry, length, and dose (Hoet et al. 2004). Kreyling et al. have reported that nanoparticles of 18 nm were absorbed in the gut wall in rats (Kreyling et al. 2011). Florence showed that positive charge of particles facilitates their uptake in the gut (Florence 2005). Jani et al. carried out a research to figure out the size effect of nanoparticles on uptake (Jani et al. 1990). They showed that the smaller the particles are, the higher the uptake is. Also it was reported that the gastrointestinal barrier is not effective for smaller particles (Ballestri et al. 2001). Chen et al. worked on nanocopper and the acute toxicity of bulk copper particles in mice (Chen et al. 2006). They found that nanocopper was more dangerous and lethal than bulk copper particles and they also reported that nanocoppers led to damage to spleen, liver, and kidney. Also the health condition of people has great impact on nanoparticle accumulation. People who have Crohn's disease, cancer, and ulcerative colitis have nanoparticles constantly in their colon tissue, while healthy people do not (Gatti 2004). Lately, it was believed that one of the reasons of Crohn's disease is the intake of high-level dietary nanoparticles (100 nm–1 μ m) and the treatment of this disease generally requires surgical intervention (Lomer et al. 2002).

Nanoparticles can enter the body via inhalation, gastrointestinal assimilation and dermal absorption. Nanoparticles also can be transported to central nervous system through blood–brain barrier or olfactory mucosa. This is very important in terms of drug delivery for damaged parts. Depending on their properties such as size, solubility, surface charge etc., they can be highly mobile in the body. Thus, their translocation tends to be remarkably fast. However, it has not been fully clarified of the relationship between the properties of nanoparticles and their circulation and fate in the body. Most of the conclusions have been drawn from animal models and it has remained as big challenge whether these models can be applied to human.

Although the effects of bioaccumulation of airborne particles on health have been established, the effects of ultrafine and nanoparticles on health have not been elucidated. Evidences indicate the translocation of iridium nanoparticles from lung to liver, spleen, heart, brain (Stern and McNeil 2008). They reported that smaller particles have a higher translocation rate.

It is particularly significant to determine the distribution of nanoparticles in the body. Once nanoparticles enter the body somehow and gain access to the blood circulation, they can be transported through body. Because of the small size, nanomaterials can run through the lungs into the bloodstream and they can reach potentially susceptible sites such as kidney, heart, spleen, and liver (Sturm 2015).

Investigations have confirmed the presence of nanoparticles in the blood and distribution of these particles in the liver, spleen, heart, and brain (Ji et al. 2006). Hillyer and Albrecht carried out a research on mice by dosing colloidal gold nanoparticles orally (Hillyer and Albrecht 2001). They showed that smaller nanoparticles (4 nm) had access to relatively distant organs like brain, while bigger nanoparticles (58 nm) accumulated in the gut. However, it is evident that nanoparticles in the circulation interact with plasma, cell, and result in coagulation of blood, but the interaction mechanism has remained unknown. Nevertheless, the interaction can have prominent effects on toxicity of nanoparticles. It has been demonstrated that bovine serum albumin reduced the toxicity of quantum dots (Lovric 2005).

The filtering process carried out by kidney, bean-shaped, which filter blood, excreting end-products of the body and regulating hydrogen, sodium, potassium, phosphate, and other ions in the extracellular fluid. Other possible nanoparticles excretion routes are via bile, sweat, and cell shedding (Nefzger et al. 1984). The question is whether the kidney has the ability to remove all nanoparticles. If nanoparticles cannot be completely removed from the body, then, however slowly they enter, some degree of bioaccumulation will occur. The results have shown that bioaccumulation is inevitable even if it is slow. There are some findings supporting the bioaccumulation. Poly (amidoamine) dendrimers of 5 nm are demonstrated to accumulate in kidney (Nigavekar et al. 2004).

2.4 Potential Health Effect of Nanomaterials

Nanotechnology is considered as the technology of the future. Most of industries including food, cosmetics, clothing, aerospace, etc. have employed nanotechnology in their products. As a matter of course, these nanomaterials enter our body as a result of exposure. As nanotechnology has been advancing and the numbers of nanotechnology-based products have been increasing, concerns relevant to their adverse effects on biological system and environment have also risen (Braakhuis et al. 2014). However, nanotechnology has great potential to create many novel materials and devices with wide-ranging applications, and a lot of questions related to the effect of nanomaterial have remained unanswered. Nanotechnology has raised a great interest related to the toxicity and environmental impacts of nanomaterials. In extreme examples, there have been various doomsday scenarios (Drexler 1986).

Scientists have been searching for the effects of exposure to nanomaterials on human health. Recent studies have indicated that some of the nanomaterials may have adverse effects on health (Viswanath and Kim 2016). The adverse effect of nanomaterials is generally related to their extremely small size. These nanoscale particles are more chemically reactive and have ability to produce a large number of reactive oxygen species. The reactive oxygen species include free radicals which are harmful to health. Additionally, their small size facilitates their entrance into the body through the skin. Unlike large-scale particles, nanomaterials are able to reach cells, tissues, and organs, and even cell mitochondria and the cell nucleus. Studies

have proven that nanomaterials may be toxic to human tissue and cause increasing oxidative stress, inflammatory cytokine production, and cell death (Oberdörster et al. 2005). Also, nanomaterials have potential to cause DNA mutation in the cells (Geiser et al. 2005). Although size is an important parameter determining the potential negative effects on health, there are some other parameters such as shape, aggregation and solubility, surface structure and charge, and the presence of functional groups of other chemicals that may cause adverse effects on health (Nel et al. 2006; Magrez et al. 2006). Studies have confirmed that shape and size of the nanomaterials have a different effect on the biological activity. For example, Pal et al. demonstrated that interaction of nanoparticles with *E. coli* is dependent on their shape (Pal et al. 2007). In another research, Journeay et al. verified that rosette nanotubes which are water-soluble structures have low toxicity to lungs because of their biological shape (Journeay et al. 2008). Different forms of nanomaterials cause different toxicological effects on health. In the light of these findings, each nanomaterial must be evaluated separately and all properties should be taken into consideration for health effect.

Humanity has limited evolutionary experience of nanomaterials with few exceptions such as nanoparticles from marine aerosols, viruses, and some engineered nanoparticles. As humanity evolved with regard to knowledge, they have taken control over the nature more effectively. Thus, they have learnt to create various nanostructures. Through the course of the handling nanoparticles, they are exposed to nanoparticles in each step. Since the area is at its early stage, there has been no comprehensive study. Thus, it is quite difficult to figure out what will be the consequences of nanotechnology. The rapid evolution of nanotechnology and its products make the task much more challenging. A variety of nanomaterials have already been manufactured and the number of products and varied structures such as buckminsterfullerene, carbon nanotubes, micelles, self-assembled monolayers, dendrimers, and aerogels seem to increase. Although it is too fast evolving area to catch up with, it is important to make a start in order to handle it safely. Numerous variations of nanoparticles and their structure probably have different effects on health and environment. This is mainly due to substantially varied properties. Below are summarized some available impacts of nanomaterials on health and environment.

Living beings have been exposed to nanoparticles which are man-made or nature-made through history. It is more likely that living beings have adapted to nanoparticles produced by natural events, but this is not true for newly engineered nanoparticles. Thus, at this point, we should be aware of the probable implications of nanomaterials. The safety of handling nanoparticles remains controversial due to lack of studies and contradictory results. Here is a summary of both in vivo and in vitro studies.

Since properties of nanoparticles are strongly dependent on their size, many biological mechanisms such as endocytosis and cellular uptake behaviors are also determined by the size of nanoparticles (Moser et al. 2016). Generally, nanoparticles can influence biological systems by modifying molecules or interfering critical processes. Thus, it becomes important to have knowledge of whether they are toxic or not. Engineered nanoparticles can lead to formation of free radicals, which are

responsible for reactive oxygen species (ROS). Free radicals can affect biosystems in various ways: DNA damage, oxidation of lipids, and inflammation. It has been observed that toxicity is size dependent as mentioned by many authors. Several toxicological studies have proved that nanoparticles smaller than 100 nm have more detrimental effects on respiratory system than larger particles (Ferreira et al. 2013). For titanium dioxide nanoparticles, it has been shown that 20–30 nm particles are considerably more toxic when it comes to respiratory health than their microparticle counterpart (>100 nm) (Vogel 2012). It has been also shown that the collection of nanoparticle on the different regions of respiratory path has been determined by the size of nanoparticles. While inhaled nanoparticles smaller than 100 nm are accumulated almost in all parts, particles smaller than 10 nm and particles in the range of 10–20 nm get deposited in tracheobronchial and alveolar region, respectively (Price et al. 2009). Also, larger particles might be safe at relatively higher doses, whereas smaller ones can be dangerous even at moderate levels (Bouallegui et al. 2017). There are exceptional cases in which it is hard to associate toxicity with particle dimensions. For example, it was observed that relatively large particles have toxicity due to their chemical properties rather than particles size. A research carried out on zebrafish using silver and gold nanoparticles has demonstrated that the behavior of silver nanoparticle was size dependent while it was not true for gold nanoparticles (Bar-Ilan et al. 2009). The size of nanoparticles is decisive in both their penetration into systems and their accumulation in organs such as liver and spleen. Larger particles were reported to lead to vascular occlusion as compared to relatively smaller particles (Fröhlich 2016).

Recently, there have been raised some concerns about whether carbon nanotubes might be hazardous due to their physical similarities with asbestos fibers. Experimental studies confirmed that some nanotubes showed asbestos-like effects. One particular concern is the potential cancer-causing risks from inhaled particles similar to those that were posed by asbestos fibers. Asbestosis is a chronic lung disease in which there is scar-like tissue formed in the lungs (pulmonary fibrosis). This fibrosis decreases the elasticity of the lungs, making breathing more difficult. Shortness of breath is the most common symptom (Poland et al. 2008; National, Health, Lung, and Blood Institute).

As mentioned above, nanotubes have many applications due to their physical, electrical, chemical, and biological attributes. However, there have been many crucial questions to be solved. Because nanotubes contain more or less impurities like metal catalyst, their biological responses may not be such desired or expected. Additionally, structural variations have also great impacts on their functionalities. Thus, precise control over nanotubes processes is particularly vital to be applied on biological systems. Because foreign substances, in this case nanotubes, are attacked by sentinel cells, there should be a way to cheat these cells by camouflage of familiar coatings. This issue also makes it quite difficult to deliver intended dose of drug to the targeted region. Besides this undesired effect, there are also numerous studies pointing out that nanotubes have toxicological effects (Poland et al. 2008). A study carried out on mouse by Takagi et al. showed that intraperitoneal injection of multi-walled nanotubes resulted in mesothelioma, a kind of cancer occurring in

mesothelium, which is covering many of internal organs (Takagi et al. 2008). The most difficult question is probably related to long-term fate of nanoparticles. Concerns have risen not only related to exposure to pure nanoparticles but also related to exposure to nanomaterial based products. Toxicity assessment of sanding dust of paint nanocomposites containing UV-Titan approved that their inflammatory response is relatively higher as compared to pure UV-Titan (Saber et al. 2012). There are some other studies showing that nanotubes cause cellular toxicity, oxidative stress, decrease in cell viability, and morphological, structural, and genetic changes in epidermal cell cultures (Ding et al. 2005).

Since titanium dioxide and carbon black nanoparticles are one of most produced chemicals, it is quite likely to be exposed to these materials in working environment. So, it is extremely important to know their potential risks. It is probable that significant fraction of nanoparticles gets deposited in alveolar region of lungs. Because clearance is low, this can result in long-term inflammation and in turn, adverse health effects. Hougaard et al. showed that 24% of titanium dioxide is detectable in lung tissue for 5 days, while 21% of particles can be detected for 25 days after exposure, which means slow clearance (Hougaard et al. 2010). Saber et al. have shown that inflammation is strongly related to the total surface area of deposited nanoparticles (Saber et al. 2011). Jackson et al. performed an experiment on mice and reported that inhaled nanoparticles resulted in increase in the lungs (Jackson et al. 2012). It has been shown that Printex 90, a form of carbon black, is able to induce reactive oxygen species in vitro (Jacobsen et al. 2008). The material may result in genetic damages (Jacobsen et al. 2011).

It has been observed that NPs smaller than 50 nm (administrated by intravenous injection) reached quickly nearly all tissues and impart potentially toxic manifestations in various tissues; on the other hand, NPs greater than 50 nm (in particular 100–200 nm positively charged particles) are readily taken up by reticuloendothelial system (RES), which refrain their path to other tissues (Gatoo et al. 2014). Although the clearance by reticulo endothelial system safeguards other tissues, it makes reticulo endothelial system organs such as the liver and spleen as the main targets of oxidative stress.

One of the most common effects of nanoparticle on biological system is the induction of oxidative stress and inflammation. There are some theories relating biological hazards to nanoparticles. Seaton et al. (1995) claimed that ultrafine particles are responsible for the inflammation in the lung (Seaton et al. 1995). The inflammation obstructs blood flow by creating blood clots. This is mostly associated with cardiovascular deaths. Another possible cell damage results from free radical production via ionization events. It has been shown that higher catalytic activity of nanoparticles led to higher production of free radicals and in turn cellular damage. This hazard can be in various ways such as genotoxicity and cell death (Rahman et al. 2002).

Buckminsterfullerene, structures of carbon atoms being about 1 nm, are expected to be highly mobile due to their tiny size. They are currently being used in drugs and targeted drug delivery, healthcare products, energy application, cosmetic products, polymer adaptations, and sporting goods (Aschberger 2012). With the extensive

usage of fullerenes, scientists started to be concerned about their potential health effects on human health. Scientists have found that these nanomaterials may have cytotoxic effects, influence embryo development, and cleave DNA or scatter rapidly to other tissues in the body (Tsuchiya et al. 1996). The nanoparticle was demonstrated to cause reactive oxygen species and peroxidation (Kamat et al. 2000). Additionally, Sayes et al. have shown that cytotoxic potential of these structures can be changed by about seven orders via surface modification (Sayes et al. 2004). However, there is no consensus for now whether buckminsterfullerenes cause oxidative stress. While Oberdörster et al. reported that the structures were responsible for oxidative stress, Zhu et al. claimed that the source of the stress is tetrahydrofuran used to make nanoparticles soluble (Oberdörster et al. 2004; Zhu et al. 2006).

Through history, it seems that human immune system has evolved for fighting against biological nanoparticles like viruses. However, it is not true for engineered nanoparticles. Engineered nanoparticles may affect such innate defense system and lead to inflammation. This is another issue to be solved since the knowledge of interaction of nanoparticles and immune receptors is not sufficient to make general and correct conclusions. Additional complexity arises when taking internalized nanoparticles by protein into consideration.

It has been shown that the toxicity and inflammatory response of nanoparticles were determined by the size of nanoparticles. Donaldson et al. reported that carbon black with the size of 14 nm was about 3 times more toxic than carbon black having 50 nm size, while it was 10 times more toxic than its counterparts of 250 nm size (Donaldson et al. 1999). Besides, Donalds et al. showed that there were almost no differences in toxicity levels of titanium dioxide and latex (Donaldson et al. 2000). There have been some *in vivo* experiments conducted to reveal the effects of engineered nanoparticles. Lam et al. demonstrated that pathological effects can last for 90 days post-exposure in mice (Lam et al. 2004).

Heavy metals also draw attention in terms of toxicology. In many hypotheses, most of attention has been devoted to chemical processes rather than physical processes. Genotoxic damage has been explained as a process in which oxidative stress through production of secondary photoelectron is linked to damage. Irradiation of material by electromagnetic radiation leads to the creation of photoelectrons. Most of photoelectrons are absorbed in bulk material, while many of electrons can escaped from nano-sized material. Thus, these escaped electrons can enter the surrounding tissue and bring about the production of reactive oxygen species.

Cellular membranes are of great importance since they enclose every living cells. They take part in many biological processes such as uptake of solid, fluid substance and ions, gases exchange, and maintenance of ionic concentration. They are responsible for cell integrity. Thus, the efficiently functioning membrane is vital for cell. The dysfunction of cell due to damage occurred in phospholipid bilayer via foreign substances such as surfactant and lipid peroxidation may be extremely dangerous for the cell. There are several mechanisms such as oxidative stress, inflammation, and protein misfolding. In literature, there have been some findings which reveal nanoparticles' effects on essential parts of cell like mitochondria and nucleus. The energy production center is mitochondria organelle, which is enclosed by two

membranes: inner and outer membrane. Being damaged by any incident, membrane degrades or destroys essential functions of the cell. Foley et al. have shown that fullerenes can cross the membrane of living cells and localize in the mitochondria (Foley et al. 2002). The size-dependent permeability of nanoparticles has been reported by Salnikov et al. (2007). They claimed that 3 nm gold particles could pass membranes of mitochondria while 6 nm gold nanoparticles could not. It has been shown that airborne particles were found in the organelle (Li et al. 2003). Another research has revealed that ambient ultrafine particles caused damage to mitochondria with increased calcium uptake and ROS production (Xia et al. 2006). A research carried out by Hussain et al. on metallic nanoparticles has confirmed that among them, silver nanoparticles are the most likely to lead to dysfunction of mitochondria (Hussain et al. 2005).

Nucleus includes DNA and damage to DNA leads to genetic mutations. Diseases resulted from these mutations can be transmitted to next generations. Thus, it is of great importance to investigate the effects of nanoparticles on DNA. Godbey has claimed that possible portals of entry of nanoparticles into nucleus membrane can be either via interaction of nanoparticles coated with phospholipids with the membrane or diffusing through pores (Godbey et al. 1999). Nanoparticle may also lead to protein misfolding and bring about dysfunction of it. When taking internally produced nanoparticles and their interaction with engineered nanoparticles into account, the scenario of their circulation and fate becomes fiendishly complicated.

Most kinds of diseases are associated with protein misfolding. The protein misfolding may cause non-functioning proteins, change their solubility or production of toxic oligomers (Cottingham et al. 2002). If the misfolding increases, highly probable are adverse consequences. It has been shown that nanoparticles have the ability to affect protein folding. Nanoparticles can disturb protein folding and the phenomenon is known as chaperone effect. Billsten et al. reported that silica particles of 9 nm can change the configuration of human carbonic anhydrase II (Billsten et al. 1997). It has been revealed that the rate of fibrillation of amyloid has been perturbed by nanoparticles (Linse et al. 2007). However, Ishii et al. showed that semiconductor nanoparticle can be stabilized and they can be transported by Adenosine triphosphate (Ishii et al. 2003). This is promising for future biomediated devices. It is clear that the available data are lacking to reveal the interaction of nanoparticles with proteins and their effect is too far to be predicted for now.

The particle size is of key importance for evaluating health hazards of airborne particles. Relatively larger particles ($>10\text{ }\mu\text{m}$) are generally caught in the nose and throat. Particles which are smaller than $10\text{ }\mu\text{m}$ get access to upper branches where body removes them by coughing, spitting, or swallowing (Jang 2012). As particle size decreases, they can travel further. Particles with size of $5\text{ }\mu\text{m}$ and smaller can reach bronchial tubes at the top of the lung. Particles which are smaller than $2.5\text{ }\mu\text{m}$ get access to alveolar portions of lungs. Although their toxicological mechanism is not clear enough to draw some logical conclusions, there are strong association between fine particles and health hazards such as lung cancer, cardiovascular diseases, asthma, and bronchitis. Particles smaller than $0.1\text{ }\mu\text{m}$ (in the range of nanoscale, $1\text{--}100\text{ nm}$) have been shown to be most harmful since they can reach

alveolar parts of lung and there are no efficient systems to remove them. Furthermore, the situation becomes worse if the particles are water-soluble. Because they can easily incorporate into bloodstream, they have access to many parts of body. If they are not soluble, they are collected by macrophages and delivered to lymph nodes. It is known that nanoparticles deposit in the alveoli, where they are predominantly cleared via normal macrophage-mediated mechanisms. However, the size and shape of nanomaterials have an impact on the efficiency of phagocytosis. In some studies, researchers showed that macrophages did not phagocytose efficiently the general nanoscale size polyethylene nanoparticles that deposited in the alveolar region (Keller et al. 2014). After the alveolar region nanoparticles go to the lung epithelium, they pass to the blood and lymph, and finally reach the cells in the spleen, bone marrow, lymph nodes, and the heart (Nurkiewicz et al. 2006). The translocation of particles is dependent on their physicochemical properties, but it is not clear whether chronic exposure leads to sufficient exposure to trigger disease (Poland 2012).

As nanotechnology evolves, the number of nanoproducts application in nanomedicine and biomedicine also increases. Since nanoparticles can be extremely small and have large surface area, they may access to any part or organ in body. During this travel, nanoparticles inside biological fluid may be covered with proteins, known as protein corona, which can drastically modify their interaction with biological systems. Corona mainly consists of hard corona, long-term adsorbed layer of biomolecules, and soft corona, relatively short-term layer. Thus, it is particularly noteworthy to shed light on the behavior of nanoparticles in biofluids. A significant point is conformation alterations of proteins during corona formation. The change can be quite strong depending on the degree of flexibility of adsorbed proteins. This can cause dysfunctions of proteins and inhibit vital processes. As the interactions are controlled by the corona surface and the identity of nanoparticle mainly is determined by the corona, the composition of corona becomes vitally important. This composition may alter considerably in some cases as it is transported from one media to a relatively different media. In such cases, while some biomolecules adsorb, some of adsorbed proteins desorb (Kaufman et al. 2007). It has been reported that protein corona may play role in cellular uptake and toxicity of nanoparticles (Debamitra et al. 2007; Walkey et al. 2014). This protein corona is chiefly responsible for interaction with cells and determining the fate, circulation in blood, distribution and cellular uptake of nanoparticles (Dobrovoiskaia et al. 2008). For drug delivery, the period of circulating in blood is of great importance. It has been demonstrated that nanoparticles with specific coronas can escape from being recognized by macrophages and this prolongs nanoparticles presence in blood (Caracciolo et al. 2015). It has been shown that recognition of particle is determined by the protein corona. Srivastav et al. have reported that ZnO with corona containing high amount of opsonin protein was easily recognized by immune cells and rapidly removed from body by inducing toxicity. Thus, the therapeutic efficacy of nanoparticle is considerably reduced (Srivastav et al. 2019). A very common way to avoid reticuloendothelial system to remove nanoparticle is PEGylation. However, PEGylation does not totally prevent nanoparticles from coronation and since the

polymer is not biodegradable, its usage is risky, especially for long-term treatment (Fadeel 2019). In order to overcome this obstacles, researchers tried many various particles. The interaction of pre-coated liposomes with immune cell were investigated (Giulimondi et al. 2019). They showed that liposomes coated with artificial corona obtained from human plasma proteins mitigate capture by leukocytes and in turn enabled extended duration in vivo. A research carried out by Cornovale et al. revealed the shape and coating-dependent cytotoxicity and cellular uptake of gold nanoparticles. Citrate gold nanospheres, tyrosine gold nanospheres, and tryptophan gold nanospheres showed the highest cell viability at 100 μ M gold nanoparticle concentration in the presence of serum with respect to gold nanospheres stabilized with cetyltrimethylammonium bromide and citrate. A significant increase in viability was observed for tyrosine gold nanospheres when these nanoparticles were pre-incubated with serum and supplemented serum. The particle shape has also strong effect on viability. While there was no significant change in cell viability for gold nanoprisms in serum-free, serum pre-incubated, and serum-supplemented conditions, a dramatic increase was observed for gold nanospheres treated with cetyltrimethylammonium bromide in serum-supplemented media (72.1%) compared with serum-free media (0%). Taking the concentration into account, in contrast to gold nanorods and gold nanocubes, it was observed that there was a sharp decrease in cell viability percentage for gold nanospheres and gold nanoprism for all three conditions. Also for all gold nanoparticle with different shapes, cellular uptake of particles is the highest in serum-free condition (Carnovale et al. 2019). It has been reported that pristine nanoparticles with no corona have a higher cellular uptake than ones with corona (Muller et al. 2018). However cellular uptake was shown to be corona-dependent. Deglycosylation of nanoparticle can enhance of cellular uptake depending on corona type (Ghazaryan et al. 2019). Cellular uptake was observed to increase for Clusterin corona and to decrease for Apoprotein A1 (Renaudin et al. 2019). Another example for toxicity is related to usage of graphene oxide. It has been reported that toxicity of graphene oxide, at low level concentration (10%), could be decreased considerably by coronation by fetal bovine serum. However, graphene oxide with high dose may induce oxidative stress and cytotoxicity (Zhang et al. 2019). Protein coating can also mitigate crystal-induced inflammation. It has been reported that Monosodium urate and monoclinic calcium pyrophosphate dihydrate crystals coated with protein decreased IL-1 β cytokine production. Silica nanoparticles were reported to adsorb strongly to cell membrane in serum-free medium while the adsorption of nanoparticles with corona has been relatively weak (Lesniak et al. 2012). However, there is no conclusive theory revealing the facts behind corona–cell interactions, and we can enjoy the fruits of corona formation by tailoring its properties. One of the most promising sides of it is that non-biocompatible nanoproducts can be biocompatible ones via coating with appropriate proteins. Hence, using appropriate coating can enhance drug efficiency and alleviate side effect of drug. The most challenging obstacle is how much control over this process can be taken. Probably in the very near future, special and quite effective drugs delivery methods are to be introduced.

2.5 Risk Assessment

It is obvious that many industries employ products based on nanotechnology in order to derive substantial benefit from it. As we already mentioned above, there are many benefits of nanotechnology. The number of nanotechnology-based products in industry has been significantly increasing. Thus, special care should be taken before these products are put on the market. So far, we have ignored the other side of coin. As nanotechnology advances, the concerns about the safety of the nanotechnology increase. There is much debate whether products of the nanotechnology pose health and environmental risks. Their small sizes and novel properties may pose significant risks to the health and environment.

Nanomaterials and their products are rapidly increasing and both human and environment are exposed to nanoparticles during manufacturing, use, and disposal of products. Thus, the exposure to nanoparticle becomes more possible. The amount of exposure can vary depending on different groups such as men, women, and workers in production, transportation, or disposal stages. To have an idea how the risk of exposure to nanoparticles is prominent, it is helpful to give some examples. Below are given some lethal incidents due to exposure.

One of the significant incidents occurred in 2006 in Germany. About 100 consumers experienced severe breathing problems after using Nano Magic, a protective glass and bathroom sealant (Glaza 2010). The manufacturer had added nanoparticles to their product (Pescovitz 2006). The content of product was not provided or explained. The Bundesinstitut für Risikobewertung (BfR) confirmed the absence of nanoparticles in the product (Elvin 2006). Later, it is claimed that the observed effects arose from tiny droplets of aerosol liquid spray which facilitate the penetration of solvent to the lungs.

Another significant incident that occurred in 2008 in a Chinese print plant resulted in seven young women having shortness of breath and excess liquid in the lungs and eventually two of them were dead. Several tests were performed on the women. Results obtained by transmission electron microscopy confirmed the presence of nanoparticles in cytoplasm and caryoplasm of pulmonary epithelial and mesothelial cells. Also, particles of about 30 nm diameter were found in the fluid surrounding the patient's lungs and it was reported that similar-sized nanoparticle were present in polyacrylic ester paste, used in the print plate, and in workplace ventilation system (Song et al. 2009). Since the data of the study were limited, it is difficult to make general conclusions (Maynard 2009). Nevertheless, appropriate workplace conditions should be implemented in order to avoid preventable incidents (Brain et al. 2010).

It is obvious that appropriate control and risk management methodologies can provide protection of workers against exposure of engineered nanomaterials. The issue of implementation of a proper method becomes extremely unforeseeable due to lack of data in the nanomaterial area. However, some measures can be taken in order to minimize exposure to nanomaterials. This can be achieved using a properly designed enclosure or containment, effective ventilation and filtration system. The

filtration should be also applied to personal protective equipment. Another point need to be considered is the case of leakage of nanomaterials or chemicals. Since some products such as baby bottles, pacifiers, and healthcare products containing nanosilver for antimicrobial activity (Chaudhry 2012) are intended for use by specific groups such as children and other vulnerable groups, the exposure assessment for these groups is also an urgent need. Although scarce, evidence from animal studies suggests that some nanoparticles might be toxic to vulnerable subgroups, such as fetuses. For instance, mice treated with titanium dioxide nanoparticles of 35 nm size have shown to have smaller uteri and smaller fetuses than untreated control after pregnant mice had been injected intravenously. Also nanoparticles were found in the placenta, fetal liver, and fetal brain (Yamashita et al. 2011).

Since the data are limited, systematic data collection of short-term and long-term exposure, manufacturing conditions, level of production, industrial applications, consumer products, and environmental fate and distribution is an urgent need (Hansen 2012).

However, there is no consensus on the best metric in terms of risk assessment; it has been agreed that comprehensive investigation should be performed to express nanomaterial in a metric such as number, surface area, or mass. Seaton et al. have discussed which metric is the most suitable for human exposure to nanoparticles (Seaton et al. 2010). The conventional dose metric is based on gravimetric measurements. For nanoparticles, it seems that particle number is more proper than particle mass. However, if particles are not uniform in terms of size, it will be complicated to compare concentrations.

It has been observed that there is a relation between toxicological response and dose by mass for some materials such as C60 and carbon nanotubes. However, it has been observed that mass concentration is not the most significant metric of exposure assessment. Owing to the lack of information related to the mechanism of nanoparticle actions and technical deficiencies, providing a suitable metric is difficult (Poland 2012). Besides mass-dependent toxicity, studies have shown that physical and chemical properties might also have toxicological effects (Loft 2012). It is recommended that the surface area of low-soluble nanomaterials is a better indicator of inflammation (Howard 2012). It has been claimed that particle number was the best metric for risk assessment while the number of functional groups has been suggested in others. The reason for varied metric suggested is the lack of information about nanomaterials and methods used. Also there is considerable debate about whether the current health and safety protocols, guidelines, animal models are feasible to make risk assessment for human (Kearns 2012).

It was suggested that case-by-case risk assessment should be carried out in order to take specific properties of specific nanomaterials into account (Kobe 2012). However, nano world has a large population in terms of structure, material, sizes, and physical and chemical variety, etc. All these parameters should be considered for risk assessment. Each of these parameters and the interactions between them make case-by-case risk assessment almost impossible unless the parameter determining outcome is not fully clear. Thus renewed methods and tools are necessary for assessment of potential risk of nanomaterials. Unfortunately, such detailed

information is lacking for virtually every type of nanomaterial or group of nanomaterials, and technical difficulties hamper the accurate measurement of nanomaterials in the workplace as well as in the environment.

There are several methods such as Swiss Precautionary Matrix, NanoRiskCat, and Control/risk banding nano tools developed to control the risk of engineered nanomaterials. The main principles of the first one are; 1- involving producers, consumers, and environment, 2- low input, and 3- to be based on the worst case of nano-specific risk (Riediker 2012). In order to assess how big nano-specific risk is, a score is derived based on information about whether the material is nanomaterial, intrinsic properties of nanomaterial, and potential human exposure and emissions to the environment. Depending the score, precautionary need is classified A (0–20) or B (bigger than 20). In case of B, available measures should be specifically revised. The matrix has a dual goal as an early warning system and self-supervision according to the chemical and environmental law. The second is presented by Hansen to support companies and regulators (Hansen 2012). The outcome of NanoRiskCat includes a short title describing the intended use and color code consisting of five dots. The first three dots refer to potential exposure while the last two dots indicate hazard potential. The third method recommended by Brouwer focuses on occupational use of nanoparticles and nanomaterial-embedded products. The outcome of the method is qualitative due to scientific uncertainty (Brouwer 2012). Control/risk banding is a simplified approach to evaluate hazard severity and exposure probability.

It is highly challenging to monitor possible health effects and interaction mechanisms of nanoparticles owing to inefficient data and characterization techniques. Risk evaluation and hazard identification become more complicated due to diversity of nanomaterials. Also many toxicological studies are *in vitro* and the prediction of effect of nanomaterials on human health may be misleading. The relation between nanomaterials and human health is not clear for now. Also which properties of nanomaterial are determining toxicity is controversial.

To summarize, as the newer nanoproducts are produced, the gap between our knowledge and their properties widens and the available data on nanotechnology and their health impact are far from conclusive. Thus, before universal applications of nanomaterials, extensive and comprehensive studies should be carried out.

2.6 Summary and Conclusion

It is clear that nanotechnology has a lot of benefits and encompasses every aspect of our lives. Since nanotechnology offers ample and exciting opportunities, it has a profound effect on our lives. Nanotechnology is fast-growing area which has many and various applications such as energy, medicine, agriculture, electronics, environmental applications, textile, and much more. However, nanotechnology might not be so safe. As nanomaterials have been evolving, the concern about the health effects of nanomaterials has increased correspondingly. Although there have been many controversial studies related to the origin of their adverse effects, there is a

consensus that nanomaterials can be dangerous. Although it is not clear that which properties of nanoparticles determine their behavior, some adverse effects such as inflammation and toxicology have been observed. The available data demonstrate that some of the engineered nanomaterials can be distributed and accumulate throughout the body and can cause toxic effects in many organs and tissues such as liver, lungs, spleen, bones, and brain. Some of these nanomaterials such as carbon nanotubes are resistant to biodegradation and therefore it is quite likely to remain in the nature or in body in case of in vivo application for a very long time. Because the number of nanomaterials and their products increases, their exposure to human and environment becomes significant. Thus, proper precautions should be followed in order to avoid the harmful effects of nanomaterials. In this regard, government agencies, industries, and research institutions should take their responsibility to prevent unfortunate consequences. Developing risk management strategies and rigid preventive procedures is an urgent need to limit the detrimental effects of nanoparticles exposure.

Hopefully, all of these adjustments and precautions will increase our awareness of the damage of nanomaterials and will significantly reduce human exposure to potentially toxic nanomaterials.

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Chapter 3

Safety and Utility of Nanomaterials on Reproduction and Development: An Update of Alternative Methods



Anna Giulia Cattaneo

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Abstract Nanomaterials offer innovative and convenient solutions to deliver drugs or genes to the embryo and fetus, improve artificial fertilization, induce in vitro differentiation of stem cells, promote controlled growth, and obtain specialized tissues and mini-organs. However, the impact of nanomaterials implies a significant risk of reproductive toxicity, whose assessment is challenging. Therefore, well-planned alternative methods, not involving mammals, are needed to reduce the burden. The double aim of this review is, on the one hand, to identify the nanomaterials that can be proficiently exploited in medicine and biology, and, on the other hand, to define those unequivocally toxic for the reproduction of vertebrates. Each section is dedicated to a set of tests for the evaluation of the effects of

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V. Kumar et al. (eds.), *Nanotoxicology and Nanoecotoxicology Vol. 1*,

Environmental Chemistry for a Sustainable World 59,

https://doi.org/10.1007/978-3-030-63241-0_3

nanomaterials on different components of the reproductive system, namely the gametes, the placental barrier, and the zygote. The side effects on the selected models seem to be mainly linked to the chemical nature, and the toxicity generally moderate. We conclude that the *in vitro* methods, alternative to *in vivo* experiences in mammals, are proficient for the assessment of the reproductive toxicity of nanomaterials, as well as for convenient testing of the possible exploitations of newer materials.

Keywords Reproductive toxicology · Nanomaterials · Alternative models · Biomedical exploitations · Perfused placenta · TransWell system · Whole Embryo · Culture · Embryonic stem cells · Zebrafish embryo test

3.1 Introduction

The impact of engineered nanomaterials on the vertebrate reproduction is double-sided: while several nanomaterials can be successful agents for theragnostics, many of them can show harmful reproductive toxicity. They can improve the artificial insemination, cross the chorion or the placenta to deliver drugs to the zygote, or, just the opposite, treat the mother and avoid the exposure of the zygote. Several nanomaterials improve the growth and self-renewal of the undifferentiated embryonic stem cells, substitute the presence of feeder cells, help the differentiation into specialized precursors, transfect and track the stem cells, and finally, apply to regenerative medicine (Chen et al. 2014).

However, because many nanomaterials have reproductive toxicity, their acceptable safety level must be documented. Thus, the assessment of reproductive toxicity of nanomaterials is very important, not only for a safe use of them in biology, veterinary and medicine, but also for a classification of environmental pollutants, products, and by-products of industry. The assessment of reproductive toxicity of chemicals with traditional methods is, however, challenging for several reasons. It requires at least two species, usually rat or mice and rabbit, and the first- and second-generation protocols require a long observational period, the sacrifice of several dams and their litters. Environmental, endocrine, and behavioral factors increase the complexity of studies.

The introduction of alternative assays, eventually as a battery, permits to explore the safety of new nanomaterials, the molecular mechanism of toxicity, and their exploitation at lower cost, as summarized in the R3 concept, that is, reduction, refinement, and replacement of the necessary tests in mammals. After a decade-long period of increasing interest, these methods became accepted in the guidelines issued by the International Council for Harmonization of technical requirements for human use (2017), which the European Medicine Agency (2017) promptly accepted. The guidelines recommended the use of alternative methods to reduce, refine, and in part replace the necessary tests “*in vivo*,” as shown in the simplified flowchart (Fig. 3.1). Recommended protocols include the use of stem cells, stabilized cell

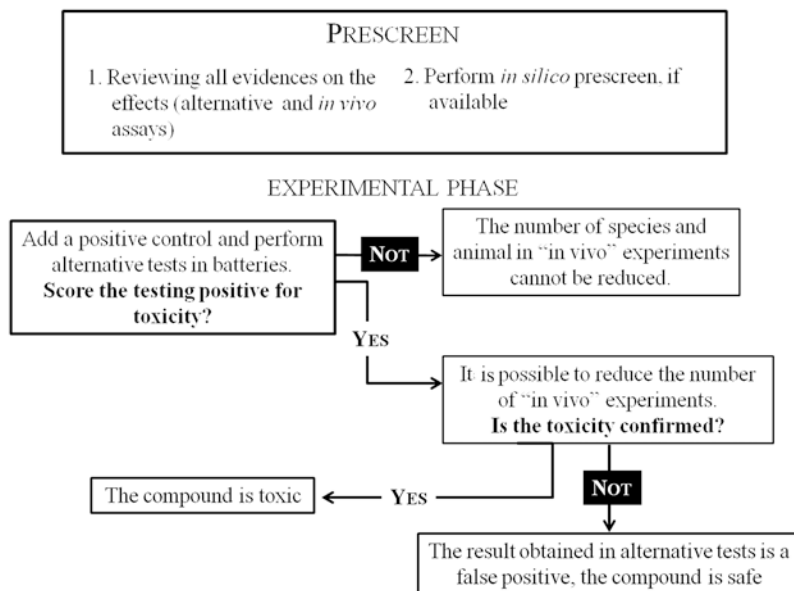


Fig. 3.1 Simplified flowchart for the use of alternative methods of reproductive toxicity, to refine, reduce, and in part replace the necessary tests “in vivo” in mammals

lines, the culture of mammal embryos still unable of independent life, and non-mammals embryonic or larval development. In this last group, the zebrafish plays a pivotal role as model species and bridges together reproductive toxicity and environmental studies. These last exceed the limits of this work; however, some recent and comprehensive review are available (Bour et al. 2015; Cattaneo 2018; Gambardella et al. 2016; Celá et al. 2014; Higashisaka et al. 2017; Revel et al. 2017; Sarmah and Marrs 2016; Taylor et al. 2012).

The implementation of alternative methods to study the reproductive toxicity of nanomaterials is recent, and no guidelines are available until now. A comprehensive and rigorous project evaluated the toxicity of ten well-characterized nano-oxides. The twelve standard *in vitro* assays focused different endpoints. The assay for the male gonad, based on mouse Leydig and Sertoli cell lines, investigated the male reproductive system, while the embryonic stem cell test focused on the embryotoxicity and differentiation of undifferentiated cells into contracting myocardial cells. Other endpoints, such as the proliferation of 3 T3 fibroblasts, are recommended to discriminate pure reproductive or developmental toxicity from general toxicity (Farcas et al. 2015). A recent protocol was specifically dedicated to the embryonic toxicity of metallic nanoparticles in the zebrafish (Pecoraro et al. 2017).

The following sections present standardized or more innovative alternative assays applied to nanomaterials and discuss the negative and positive aspects on reproduction and development.

3.2 In Vitro Exposure of Sperm and Other Cells of the Male Gonad

The male gonad contains epithelial cells developing into gametes, supportive cells, and stromal cells. The precursor of gametes and the mature gametes are particularly sensitive to toxicants, because they lack highly efficient systems to counteract the stressors. Wang et al. (2018) recently reviewed this issue. Fig. 3.2 summarizes the experimental systems to test in vitro the reproductive toxicity on male gonads. The exposure of male gametes to nanomaterials can be accidental or intentional, for contraception or fertility improvement. Because the spermatozoa are emitted outside the body, they are easily collected and used for artificial insemination of human and livestock, which represents a remarkable reason of interest. Several nanomaterials can successfully help the selection and manipulation of fertile sperm, but it is necessary to assess their safety, the sperm being very sensitive to chemical aggression.

Among useful and safe nanomaterials, we cite customized nano polymer (TransFect) and halloysite clay nanotubes, which were used for transfection of bovine sperm and generation in vitro of well-developing embryo, carrying the paternal mutant and able to implant in the uterus (Campos et al. 2011). Mesoporous silica, iron-based magnetic, and lipid core nanoparticles were also safe for sperm, and proposed for improved transfection (Barkalina et al. 2013, 2015; Caldeira et al. 2017; Cortesi et al. 2014; de Castro Jorge Silva et al. 2017; Kim et al. 2010; Vasquez et al. 2016). Organic nanoparticles loaded with melittin acted as protective agents against the spread of HIV through infected sperm. Melittin, a bee venom highly

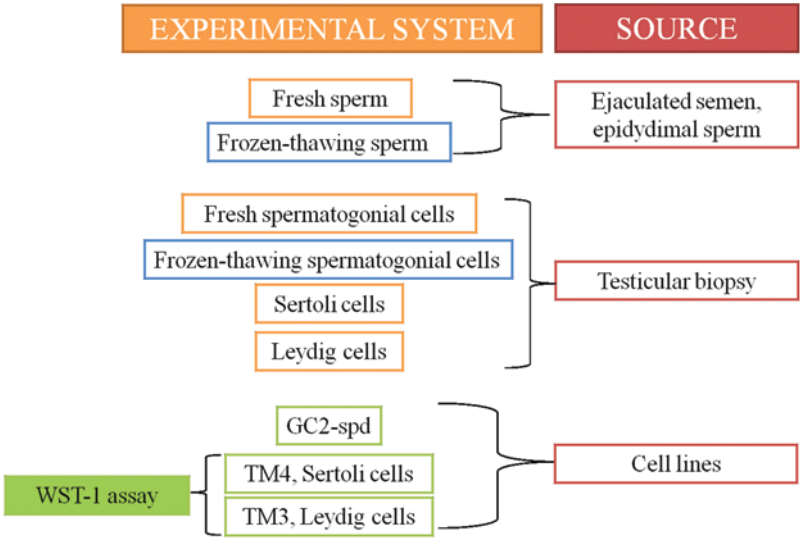


Fig. 3.2 Experimental systems to test in vitro the reproductive toxicity on male gonads

toxic for the gametes, was instead safe if loaded into nanoparticles at a therapeutic dose four times lower than the cytotoxic one (Jallouk et al. 2014).

In assisted insemination, the sperm manipulation, especially the frozen-thawing procedure, greatly reduces the quality of semen and the reproductive success. In addition, in several species of livestock, the commercial value of the offspring is influenced by the sex. Several methods allowed sex-sorting and sperm selection on the basis of their quality, while their wide utilization will wait for expensive standardization (Barkalina et al. 2016; Rath et al. 2015). In this contest, golden, magnetic, and plasmonic nanoparticles have been used in pioneering experiments. Some of them selectively entered only the sperm in which the acrosome was activated, and others helped the separation of labeled sperm (Barchanski et al. 2015; Farini et al. 2016; Odhiambo et al. 2014; Vasquez et al. 2016).

Quantum dots are semiconductor nanostructures that emit light at different wave lengths in relation with their size. They can be internalized in sperm, in such a way for labeling and tracking it during *in vitro* fertilization. Unfortunately, the quantum dots, as their dissolved components, retained in certain experimental conditions a high toxicity (Akhavan et al. 2016; Feugang et al. 2012).

The spermatogonia, precursor of sperm, represent a reserve of male gametes, and can evolve *in vitro* into mature and fertilizing sperm. Also in this case, however, the procedures necessary for conservation frequently reduce the sperm to a number below the minimum required for a successful assisted insemination. A scaffold of carbon nanotubes or nanofibrillar electrospun, acting as an artificial basement membrane, improved the production of fertile cells. The non-univocal success of this method was probably consequent to the mitochondrial damage documented in a cell line representing the spermatocytes, an intermediate stage of maturation of male germinal cells (Eslahi et al. 2013; Rafeeqi and Kaul 2010; Shakeri et al. 2013; Xu et al. 2016a).

Single-walled carbon nanoparticles and graphene oxide seemed to be safe for the sperm, but spermatogonial cells were more sensitive to the toxicity of graphene (Asghar et al. 2016; Hashemi et al. 2016). Other nanoparticles were clearly toxic to the sperm *in vitro*. In mammals, the fullerenols acted as antioxidant agents (Murugan et al. 2002), while silver and gold nanoparticles were moderately toxic only at doses higher than 10 µg/ml for gold, 125–500 µg/ml for iron (Moretti et al. 2013; Taylor et al. 2012, 2013; Terzuoli et al. 2012; Tiedemann et al. 2014). Titanium dioxide nanoparticles reduced the integrity of membrane and desoxyribonucleic acid (DNA) of buffalo sperm. The nanoparticles entered the cell and adhered to the cell membrane along the tail and to the head. The viability was compromised at high doses only (Pawar and Kaul 2014). The nanoparticles of cerium dioxide, at 10 µg/ml, were unable to enter the human sperm or reduce viability, but damaged the DNA. At high concentrations (100 mg/ml), the nanoparticles accumulated in the cytoplasm, reduced the fertility, and induced genotoxicity through oxidative damage (Préaubert et al. 2016, 2018). Braydich-Stolle et al. (2005, 2010) studied the toxicity of a panel of metal nanoparticles on the precursor spermatogonia. The toxicity was dose-dependent for all the nanoparticles tested, those of silver being the most toxic, and molybdenum trioxide the least toxic. The corresponding soluble salts were not

toxic, therefore, the dissolution was not considered to be responsible for the side effects. Nanoparticulated zinc oxide showed dose-dependent lethality in human sperm after in vitro exposure, and caused DNA damage and arrest in S-phase of a spermatocytes cell line (Liu et al. 2016b; Barkhordari et al. 2013).

Endocrine disruptors were tested with a recent assay, involving two cell lines, each of them representing the Leydig and the Sertoli cells (Farcal et al. 2015). These are two types of testicular, non-germinal cells, important for the function and architecture of the male gonad. The Sertoli cells, responsive to the pituitary follicle-stimulating hormone, support and nourish the maturing gametes and build up the blood-to-testis biological barrier, and the Leydig cells, positioned in the space between the tubules, secrete the testosterone. The golden nanorods damaged the blood-to-testis barrier through a damaged mitochondrial pathway of glycine. Altered mitochondrial membrane permeability and transmembrane potential were observed together with disrupted expression of several proteins bound to membrane function (Xu et al. 2014a). Zinc oxide and silver nanoparticles were the most toxic against these cells. At low concentration (10 µg/ml), the zinc oxide nanoparticles were lethal (Farcal et al. 2015); at sublethal doses, they entered the cytoplasm and the nucleus, reduced the membrane integrity and the viability, and increased the apoptosis (Han et al. 2016b). Apoptosis and death of Sertoli cells followed the oxidative stress caused by the exposure to silver nanoparticles or to nanocrystals of copper sulfide, which produced photothermal and photodynamic lethal effects, in agreement with what was observed in vivo (Han et al. 2016a, b; Liu et al. 2015).

Titanium dioxide nanoparticles gave contrasting results, in studies on Sertoli and Leydig cell lines. In the tiered protocol adopted by Farcal et al. (2015), the safe concentration was lower than 100 µg/l. Other studies, however, demonstrated a dose-dependent increase of mitochondrial damage in the Sertoli cell lines, with apoptosis and death. The suggested mechanism of cytotoxicity seemed to be mediated by the calcium-regulated cascade inducing inflammatory cytokines (Hong et al. 2016; Ye et al. 2017).

The carbon black, which slightly induced the aromatase expression in Leydig cell lines without affecting vitality, was a fair endocrine disruptor (Komatsu et al. (2008).

Organic nanoparticles, functionalized with the follicle-stimulating hormone and loaded with superoxide dismutase, counteracted the strong oxygenic stress produced by hydrogen peroxide in the Sertoli cell lines (Snow-Lisy et al. 2014).

3.3 In Vitro Exposure of Eggs and Follicular Cells of the Female Gonad

The functional female gonad is characterized by the periodical maturation of one or more ovary follicles. The immature follicle contains the gamete in a crown of granulosa cells. The mature follicle (antral) is a cystic formation, in which the

granulosa cells differentiate into a layer of periantral cells, delimiting the cavity filled with a fluid, and a cumulus, inside which the egg is still crowned by cells (*corona radiata*). At this stage, the cells have acquired sensitivity to the follicle-stimulating hormone, secreted by the pituitary and necessary for final maturation and ovulation. Alternative tests for female fertility include test on isolated follicles or stabilized cell lines, such as those derived from the Chinese hamster ovary, the human granulosa, and the cancerous ovarian cells.

The isolated follicles and the cumulus and oocyte complex permit to follow the maturation of the female gamete and of its environment, to produce the zygote, and study the artificial fertilization. In mammals, the female gamete matures and is fertilized inside the body; therefore, its collection for in vitro studies requires more manipulation than that necessary for the mature sperm. The granulosa cells surrounding the follicular fluid form the multilayered cumulus–oocyte complex and play a pivotal role in reproduction. The periantral cells secrete the follicular fluid, regulate the maturation of follicles, the ovulation, and the formation of corpus luteum. The cumulus cells regulate instead the maturation of the egg. In addition to methods based on egg or ovary cell lines, in vitro testing of the granulosa cells, the mature cumulus–oocyte complex, and the incubation of the entire follicle with the fluid are also useful (Fig. 3.3). The collection of undamaged oocytes greatly improved with the introduction of assisted reproductive techniques (Langbeen et al. 2015).

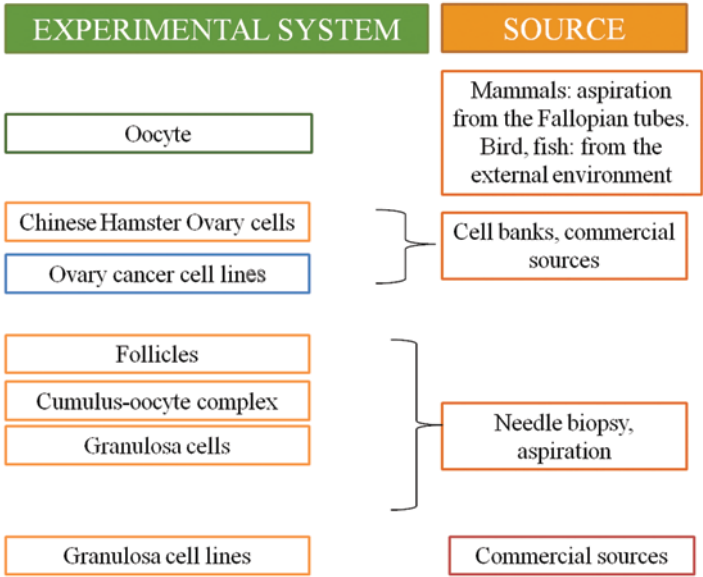


Fig. 3.3 Experimental systems to test in vitro the reproductive toxicity on female gonads

3.3.1 Collection of Oocyte and Follicular Cells of the Female Gonads

The collection of the oocyte is easy in animals with extracorporeal fecundation, such as fish, amphibians, and birds, or non-vertebrates. In mammals, the eggs must be instead aspirated from the fallopian tubes, a poorly invasive method usually applied to woman, or picked-up with a needle from a pre-ovulatory follicle.

The granulosa cells and the cumulus–oocyte complex are only available with puncture and aspiration of mature follicle. (Arashiro et al. 2013). The methods in use differ widely: those preferred in humans include an ultrasound-guided puncture, or a laparotomic procedure, generally necessary to obtain gametes for the artificial fecundation. In livestock and laboratory species, the ovariectomy is more common. The ovaries are freshly collected after death and safely transported to the laboratory. To warrant enough yield of reproductive tissue and cells, the ovulation is stimulated with injection of gonadic tropins shortly before the sacrifice. For experimental purposes, the cumulus–oocyte complex is aspirated from the antral follicles, better if the granulosa is three layered. After the isolation, the granulosa cells require purification on a gradient to avoid the contamination with blood cells (Chilvers et al. 2012; Ferrero et al. 2012; Quinn et al. 2006). Once isolated and purified, different types of cells can also be cultivated, stabilized, or immortalized, to obtain cell lines comparable to those previously cited and commercially available.

In brief, several alternative methods are available to exhaustively study the ovary function in vitro, all well standardized. They have, however, more limits in comparison with methods in use to study reproductive toxicity in the male. The low yield of isolation procedures requires previous hormonal stimulation in vivo, while the necessary higher manipulation of the functional unit, that is the follicle and its component, imposes the presence of skilled personnel at the laboratory. Ethical limits reduce the availability of experimental material from humans.

3.3.2 In Vitro Exposure of Different Types of Ovary Cells

The first study on the safety of nanomaterials for ovary cells was conducted by Bourrinet et al. 2006, in a complete preclinical study that aimed to ascertain the eligibility of super paramagnetic iron oxide nanoparticles for clinical aims. The nanoparticles were safe for the endpoint considered, the induction of chromosomal aberration in the chinese hamster ovary cells. Further studies confirmed the low toxicity of these materials in Chinese hamster ovary cells and granulosa cells, related to the intake inside the cells. Protective coating, such as with dextran, bovine serum albumin or polyethylene glycol, improved the biocompatibility and safety of nanoparticles (Hanot et al. 2015; Pöttler et al. 2015, 2016).

Other oxides inhibited the growth of Chinese hamster ovary cells acting on selective genetic pathways (Liu et al. 2017). The effect of cerium oxide nanoparticles

differed in murine mature cumulus–oocyte complexes and in those obtained after hormonal stimulation from prepubertal goats, exposed during maturation. In the first case, decreased fertilization rate and DNA damage followed the intake of nanoparticles by the granulosa cells and the zona pellucida of the egg. In the ovine cumulus instead, the nanoparticles entered the granulosa cells, not the oocyte, and increased the growth and maturation of the zygote, reducing the oxidative stress (Ariu et al. 2017; Préaubert et al. 2016). Arsenic trioxide is used for the chemotherapy of lymphoma, and the nanoparticulate formulation performed better than the traditional drug to protect the female fertility (Ahn et al. 2013).

The toxicity of gold nanoparticles seems to be low for the granulosa, while those of silver are moderately toxic and inhibit the cumulus expansion, the oocyte maturation, and second meiosis (Han et al. 2016a; Stelzer and Hutz 2009; Tiedemann et al. 2014). The results strictly depend on experimental conditions. Organic coating, protective against dissolution, quenched the high toxicity of quantum dots, which reduced the fertilization rate of cumulus–oocyte complexes. The fluorescent signal was high in the cells of mature follicles and in the sub-plasma membrane of oocytes, but weaker in the cells of the cumulus (Feugang et al. 2015; Hsieh et al. 2009; Xu et al. 2012, 2016b).

Among “soft” nanoparticles, the polymeric polyethylene glycole-poly lactide methyl ether reduced the viability of granulosa cells only at high doses and altered the secretion of progesterone and estradiol. However, this result was not exclusive to nanoparticles because the non-particulated polyethylene glycole behaved similarly (Scsukova et al. 2017). Nanoparticles conjugated with follicle-stimulating hormone or its derivative follicle-stimulating hormone₃₃ (ovarian cancer specific receptor), loaded with paclitaxel, were more precisely addressed to the tumor cells and more efficient was the paclitaxel alone, also in nanoformulation (Fan et al. 2014; Zhang et al. 2009). Lipid-core nanoparticles were safe for cumulus–oocyte complex; if loaded with melatonin, it reduced oxidative stress and enhanced meiotic maturation, cleavage, and blastocyst production (Remião et al. 2016). The carbon black reduced the aromatase expression and the production of estrogen (Simon et al. 2017),

From these considerations, the toxicity of nanomaterials on the reproductive components of the ovary seems to be low or moderate. In some cases, as drug or drug carriers, they performed better than the corresponding traditional formulation.

3.4 The Placenta: A Differentiated Mother-to-Fetus Biological Barrier in Mammals

The most useful model species for predicting toxicity in humans, as well as the subjects of artificial fertilization *in vitro*, belong to mammals. The development of mammals is unique because of the presence of a chorioallantoic placenta along the fetal period. This powerful biological barrier is selective for several solutes and

particles, and therefore protects the zygote without impeding the necessary exchanges with the mother.

The placenta develops in two times, beginning with a choriovitelline placenta, replaced by the well-vascularized chorioallantoic placenta, and when the zygote needs higher oxygen supply. Species with short pregnancy, such as mice and rat, develop the chorioallantoic placenta for a very short time. This organ is substantially different in humans and in the most popular model species and recommended models for chemical toxicity in vivo, such as rodents, rabbits, and minipig. The histological features, known from decades, differ between families and are conserved inside the orders of mammals (Enders 1965, 2009; Enders and Blankenship 1999). The histology and permeability of the placentas in livestock have been recently reviewed and compared (Furukawa et al. 2014). Several details are reported in Table 3.1 and Fig. 3.4.

3.4.1 Alternative Models to Evaluate the Transport Across the Placenta

The alternative models of the placenta transport filled the gap due to the absence of affordable models in vivo to predict toxicity in humans. We discuss here firstly the method of implementing the perfusion of placenta ex vivo, which offers several advantages. It applies to all theria that expel the organ at the end of the delivery, is not invasive, is highly standardized, and not too expensive. The main limit is that the methods apply only at the placenta at the end of pregnancy. Briefly, the placenta is maintained in a protected environment, with controlled temperature and humidity. Catheters are inserted in the chorionic artery and vein, that represent the fetal district, and others are introduced in the villous space, at the maternal surface. The

Table 3.1 Type of chorioallantoic placentas in humans and different laboratory species, or livestock, with a list of most important features

Species	Type	Maternal blood	Fetal blood	Layers
Pigs, ruminants, horses	Epitheliochorial	Endothelium, basement membrane	With endothelium	Columnar trophoblast applied to endometrial epithelium
Carnivores	Endotheliochorial	With endothelium	With endothelium	Syncytiotrophoblast, discontinuous cytotrophoblast
Humans, cynomolgus	Hemomonochorial	Sinus	With endothelium	Syncytiotrophoblast with knots, discontinuous cytotrophoblast
Rabbit	Hemodichorial	Sinus	With endothelium	Syncytiotrophoblast and cytotrophoblast
Rat, mice	Hemotrichorial	Sinus	With endothelium	Double layers of syncytiotrophoblast and cytotrophoblast

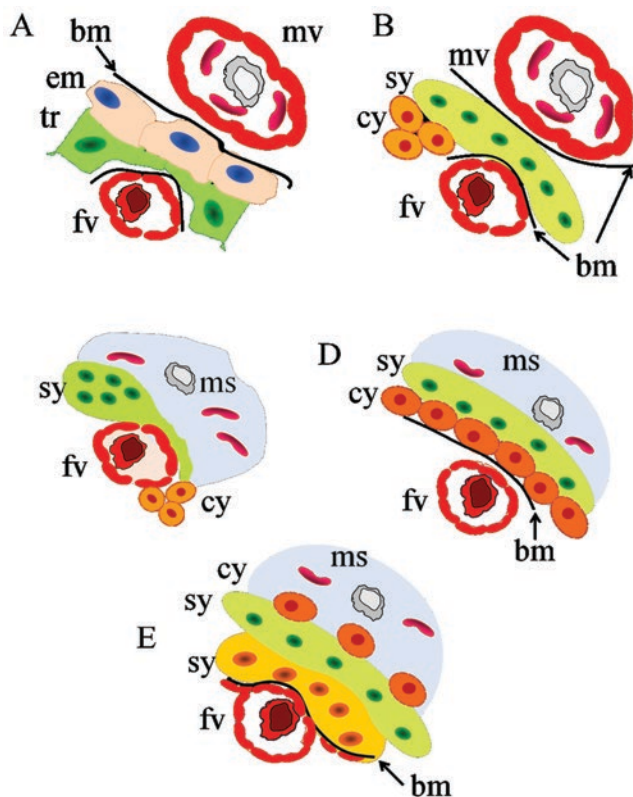


Fig. 3.4 Schematic comparison of different histologic features of the placenta in mammals. In the chorioallantoic placentas (A and B), a complete endothelium with a basement membrane (bm) limits the maternal vessels (mv). The maternal sinus (ms), lacking the basement membrane substitutes the maternal vessels in the choriovitelline placentas (C). The epitheliochorial placenta (A) retains a layer of maternal endometrial epithelium (em) in direct contact with the non-syncytium trophoblast (tr). In the endotheliochorial type (B), the discontinuous cytotrophoblast (cy), interposed to the syncytiotrophoblast (sy), replaces the endometrium. This disposition of layers is similar in the choriovitelline emomonochorial placenta (C, upper left), in which the maternal sinus (ms) replaces the maternal vessels and their basement membrane. The dichorial type (C, upper right) has a complete layer of cytotrophoblast interposed between the maternal and fetal compartments, and the trichorial type has a second layer of syncytiotrophoblast (C, bottom). The fetus vessels contain immature, nucleated red cells

Abbreviations

bm basement membrane, *cy* cytotrophoblast, *em* maternal endometrial epithelium, *fv* fetal vessel, *ms* maternal sinus, *mv* maternal blood vessel, *tr* trophoblasts, *sy* syncytiotrophoblast

flux, from the maternal district to the fetal and vice-versa, is constant, as are the temperature, pH, dissolved gas, and pressure of perfusion (Fig. 3.5). For an exhaustive description, see the works of May and coworkers (2009) and that of Grafmueller et al. (2013).

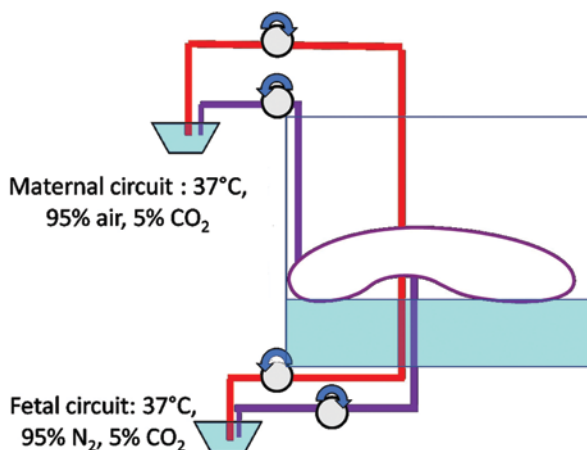


Fig. 3.5 Schema of the perfused placenta ex vivo. The placenta, expelled at the end of the pregnancy, is perfused through catheters, inserted in the chorionic artery and vein, that represent the fetal district, and in the villous space, at the maternal surface. The flux, from the maternal district to the fetal and vice-versa, is constant, as are the pressure, temperature, humidity, pH, and dissolved gases

Abbreviations: CO₂: carbon dioxide; N₂: gaseous nitrogen, °C: centigrades

Another method implements the TransWell®, a customized two-compartment system for growing cells. Two vials are inserted one inside the other. A semi permeable membrane closes the flat bottom of the inner vial. In transplacental studies, the inner vial represents the fetal vessel, the outer the maternal venous sinus. Two types of trophoblast-derived cells could develop as a confluent monolayer on the flat bottom of a TransWell®: the stabilized cell lines are representative of the labyrinthine trophoblast cells of rat, and those representative of third-trimester trophoblasts, derived from a human choriocarcinoma. When grown as a monolayer, the trophoblast cells do not form syncytium, only the tight junctions are well developed. It is also possible to grow them in multilayer (TransWell® system B). In this case, the bilayer develops after 5 days, and 10% of cells are multilayered at day 7 of culture, with initial syncytium. The system was optimized to study the transport of nanomaterials across the placenta (Bode et al. 2006; Cartwright et al. 2012; Sood et al. 2011). The presence of a monostratified cell layer with tight junctions has been confirmed by protein staining and by the measurement of transepithelial electric resistance, higher than 30 Watt per square centimeter (Cartwright et al. 2012). The monolayer can be permeable to nanomaterials, allowing a mono- or bi-directional crossing, or can accumulate several compounds, unable to cross the barrier. The absence of nanomaterials in the outer compartment (the “fetal” one) does not exclude the presence of dissolution products. On the bottom of the outer chamber, layers of other types of cells, such as stem cells, precursor, and fibroblasts, simulate the fetal tissues (Fig. 3.6).

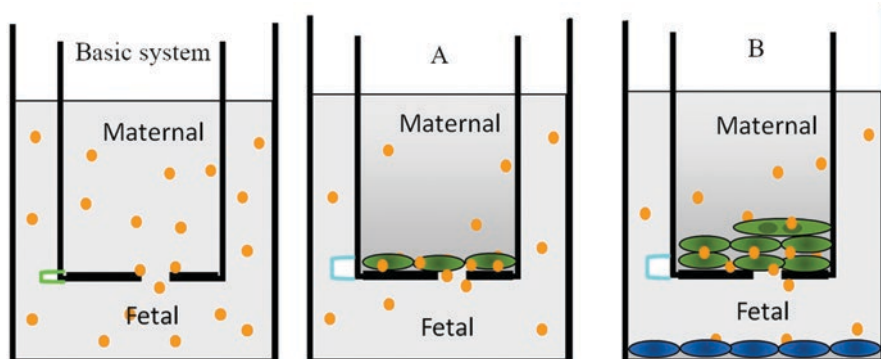


Fig. 3.6 Labyrinthine trophoblast cells (BeWo) in a TransWell® system. The basic system (A) is a two-compartment system for growing cells, composed of two vials inserted one inside the other. A semi permeable membrane closes the flat bottom of the inner vial that represents the fetal vessel. The cells, representative of third-trimester trophoblasts, grow as a monolayer in the inner vial within 5 days. A transepithelial electric resistance equal to or higher than 30 Watt per square centimeter is the signal of a complete monolayer, with well-developed tight junctions. Longer culture time, up to 9 days, is necessary to obtain a bi- or even a partial multi-layered trophoblast, sometimes with initial syncytium (C)

3.4.2 Nanomaterials and Alternative Models of the Placenta

The studies on the transport across the perfused placenta documented that polystyrene nanoparticles accumulated in the syncytiotrophoblast through an energy-dependent mechanism. The transport across the barrier was polarized, being more rapid from the maternal to the fetal environment than in the opposite direction. The accumulation of nanomaterials inside the placenta did not affect the viability of the explants, and only the nanomaterials with basic functionalization caused fluid leakage (Graefmueller et al. 2015a, 2015b; Wick et al. 2010). However, in vitro exposure of trophoblast cells to acidic polystyrene nanoparticles revealed a certain grade of cytotoxicity, as increased expression of apoptosis markers (Huang et al. 2015). The same is true for exposure to copper nanoparticles of an immortalized trophoblast cell line, in which the compound induced apoptosis, necrosis, and arrest of the cell cycle. The IC₅₀ after 48 and 72 hours of exposure was assessed at 20 and 10 µg/ml (Zhang et al. 2018). Silica and gold nanoparticles accumulated in the trophoblast or in the perfused placenta. However, only the former crossed the barrier by 5%, and the latter ones did not reach the fetal environment during the observational time of 6 hours. Studies in cells cultured in vitro confirmed these results (Myllynen et al. 2008; Poulsen et al. 2015). Polystyrene nanoparticles crossed the BeWo cells in TransWell® A without signs of cytotoxicity (Cartwright et al. 2012). Other nanoparticles tested with the TransWell® methods involved the clinical exploitation of new materials.

The super paramagnetic nanoparticles of iron oxide are powerful probes approved for magnetic resonance imaging; the cobalt and chromium alloy nanoparticles can

be released from orthopedic prosthesis, and the organic nano carriers of polylactic acid are potentially useful for drug transport across the placenta. The iron oxide nanoparticles, neutral or functionalized with anion, crossed the barrier, but disrupted the tight junctions and induced apoptosis. Cationic nanoparticles accumulated in co-cultured cells (Faust et al. 2014; Müller et al. 2018).

In TransWell® B, the cobalt and chrome nanoparticles, at low concentrations, did not cross or alter the barrier permeability; however, the upper layer of BeWo cells showed signs of impaired lysosomal function and autophagosomal clearance. The DNA was damaged, with double- or single-strand breaks and tetraploidy (Parry et al. 2010; Sood et al. 2011). The nanoparticle-free media collected in the outer compartment damaged neural cell lineage, which differentiated into an excess of glial astrocytes and endangered neurons (Hawkins et al. 2018). Some organic nanoparticles were instead safe, able to cross the barrier from mother to fetus, and could efficiently carry and release molecules, such as dexamethasone, digoxin, and antiepileptic drugs. These results open a promising perspective for prenatal care (Ali et al. 2013; Albekairi et al. 2015; Lopalco et al. 2015).

Ilekis et al. (2016) reviewed the mechanisms of placental permeability to potentially toxic agents, including nanomaterials.

3.5 Embryonic Exposure and Embryotoxicity

Once the nanoparticles have crossed the placenta, whenever possible, they meet the zygote, the final product of the reproductive process. Therefore, direct toxicity tests of on in vitro developing whole embryos can give important information on the protection by the placenta.

Living mammalian embryos for in vitro studies can be obtained in two ways: from eggs fertilized in vitro, or with the whole embryo culture test. The former assay can study very early zygote only, during the preimplantation period, while the latter requires the necessary sacrifice of the mother. This poses ethical questions; therefore, the studies in vitro with whole mammalian embryos are few and usually limited to mice and rats. The method, while not properly alternative to in vivo experiments, nevertheless bridges the gap between in vitro and in vivo studies and agrees at least with the principles of reduction and refinement.

A valuable alternative test is the zebrafish embryonic test, which meets all the three main principles of reduction/refinement/replacement concepts. The many advantages of using this small fish will be discussed in a dedicated section; here, it is enough to mention that the zebrafish embryonic test has good predictability in mammals and humans (Ball et al. 2014; He et al. 2014).

Another way to study in vitro the embryonic toxicity is the embryonic stem cells test. Undifferentiated, pluripotent cells lines are obtained from the inner cell mass of the 3.5-day mouse blastocyst, or embryos of corresponding age in other species, such as bovine and humans. Genschow et al. (2004) validated the test for the European Centre for the Validation of Alternative Methods. They exposed cells to

potential embryo-toxic agents for a period of 10 days and observed the spontaneous differentiation into beating cardiomyocytes. However, the method was subsequently modified in different laboratories, changing the endpoint or selecting more mature stem cells, such as mesenchymal or endothelial. Recent reviews on this matter are available (Brannen et al. 2016; Das et al. 2016; Handral et al. 2016). Table 3.2 summarizes several recommended protocols. The most important characteristics, and the results in nanotoxicity, are discussed in next subsections.

3.5.1 *Culture of Early Mammal Embryo*

The early zygote of mammals, obtained by artificial fertilization of a mature egg, can evolve outside the maternal body until the blastocyst stage, that is, the preimplantation period, ending with the “hatching” of the animal pole from the pellucida zone. The early zygote development proceeds from the fecundated egg into the two-cell stage, the morula and the blastocyst, where the polarity of the zygote is established between the animal pole, where the embryo and the future placenta will develop, and the opposite pole, evolving into extra-embryonic membranes. The overall viability, the blastocyst rate, and cell number are the endpoints most frequently studied. The hatching rate, the cavitation rate, the expression of markers of maturation, such as trophectoderm-associated genes, or of oxidative stress and apoptosis markers are other indicators of safety/toxicity. If transferred in vivo, in optimal conditions, the blastocyst starts implantation and generates a new individual: this is the endpoint of the methods for artificial reproduction. Low implantation and high resorption rate, or poor vitality and performance of newborns, give a measure of risk hazard in toxicology.

Several studies tested the effects of different types of nanoparticles in this model. Polystyrene was safer than polyacrylonitrile, and both inhibited the hatching of mouse blastocyst (Fynnewever et al. 2007). Instead, the organic nanoparticles of polylactic-co-glycolic acid seems to be safe to the in vitro developing mouse embryo. The early embryo accumulated labeled nanoparticles, which persisted in all preimplantation stages. Moreover, after the transfer in a healthy female, the development proceeded normally until the delivery of healthy offspring (Kim et al. 2018).

The Quantum Dots induced apoptosis and reduced the cell number in the blastocysts (Chan and Shiao 2008; Hsieh et al. 2009), as did silver nanoparticles, that reduced the successful implantation and development when embryos were transferred into the uterus (Li et al. 2010). Chitosan nanoparticles were toxic for exposed morula, while nanocapsules with a lipid core, loaded with antioxidant agents, such as tretinoin and melatonin, had positive effects on the development of bovine blastocysts after artificial fertilization (Gomes Lucas et al. 2015; Komninou et al. 2016; Remião et al. 2016). Golden nanoparticles were instead safe, according to Taylor et al. (2014). These evidences assume a special relevance in the perspective of possible exploitations of nanotechnology to improve the success and performance of artificial fecundation techniques.

Table 3.2 Recommended protocols for alternative assays for the in vitro assessment of embryonic toxicity

Citation	Study	Assays	Endpoints and predictability
Farcas et al. 2015	FP7-MARINA project	TM3 and TM4, EST	Toxicity assay for the epithelial Leydig and Sertoli cells in testis or embryo stem cells exposed to nanoparticles
Genschow et al. 2004	Validation for the ECVAM.	Rating embryotoxicity in D3 and 3 T3 cell lines.	Assessment of three endpoints, IC50 in D3, IC50 n 3 T3, and ID50 in D3.
Brannen et al. 2016	Publication	EST	Pluripotent embryonic stem cells evolving into differentiated cells Agree with embryo-fetal developmental toxicity
Piersma 2011	RIVM report 340,700,005/2011	WEC and EST ZET: GMS.	Improvement with gene expression profiles Defining effective (or toxic) dose. Agreement with studies “in vivo”
Brannen et al. 2016	Publication	WEC (rodents)	Decorticated embryos cultivated in vials, development from early to late somite state
OECD (2013), Test No. 236.	Guidelines	FET/ZET	LC50, fertilization rate 24–96 hrs: Coagulation of the embryo, lack of somite formation, non-detachment of the tail. 48–96 hrs: Lack of heartbeat
Ducharme et al. 2013	Validation of OECD test no. 236 (FET/ZET)	ToxPi and a teratogen ratio (LC50/ LOADED)	Lethality, embryotoxicity
Busquet et al. 2014	OECD validation	ZET	Intra- and inter-laboratory reproducibility
Beekhuijzen et al. 2015	Publication	ZET	Classification of teratogenicity index, comparison of endpoints and methods for calculation
Brannen et al. 2016	Publication	ZET	Embryonic development from early zygote to adult
Pecoraro et al. 2017	Protocol	ZET	Toxicity assay with identification of specific biomarkers, applied to metallic nanoparticles
NIEHS 2000	FETAX background review document	Embryonal development in <i>Xenopus laevis</i>	Teratogenicity, embryonic and larval toxicity

(continued)

Table 3.2 (continued)

Citation	Study	Assays	Endpoints and predictability
van der Burg et al. 2015	Chem screen	In silico integrated strategy.	Improving prediction and reducing number of experiments through integrated in silico test and databases, in vitro minimal essential screening, and in vivo testing. 74–94%, agreement with other studies

Abbreviations: *bIVF* Bovine in vitro fertilization assay, *bIVM* Bovine in vitro maturation assay, *D3* embryo stem cells, differentiating into cardiomyocytes; *ECVAM* European Centre for the Validation of Alternative Methods, *EST* Embryonic stem cell test, *FET* Fish Embryo Acute Toxicity, *GMS* General Morphology Score, *Ishikawa* Ishikawa cell test; *ID50*, *IC50* dose, or concentration inhibiting the growth of the sample by 50%, *LC50* concentration inducing 50% lethality in the sample, *LOADED* Lowest Observed Adverse Developmental Effect Dose (excluded lethality), *MEPA* Mouse embryonic peri-implantation assay, *NIEHS* National Institute of Environmental Health Sciences, *OECD* Organisation for Economic Co-operation and Development, *RIVM* Ministry of Health, Welfare, and Sports of Netherland, *ReProGlo* Luciferase activity of promoter-driven reporter plasmid in mouse embryonic stem cells (Wnt signaling), *3 T3* mouse embryonic fibroblasts, *TM3* stabilized Leydig cell line, *TM4* stabilized Sertoli cell line, *ToxPi* Toxicological Priority Index program, *WEC*: Whole embryo culture, *ZET* Zebrafish Embryonic Test

3.5.2 Whole Embryo Culture

The mammals whole embryo culture is a validated test for embryotoxicity studies (Scientific Committee on Consumer Products 2007) that evaluates the development from the early somite stage (3–5 somites) to a late stage (21–25 somites). The rat is sometimes preferred to the mice because the embryos are larger and the necessary manipulation easier; in this species, the preimplantation process requires 48 hours to be complete. During this period in which the embryo does not depend on the placenta, morphogenesis and organogenesis take place. Central nervous system, heart, vertebrae, limb buds, and craniofacial structures develop in vitro with a similar rate to that of in vivo. The requirement of oxygen increases rapidly during the late gastrulation and early neurulation; therefore, the embryo, partially deprived of membranes, is cultured in an incubator with increasing concentration of oxygen. The morphogenetic process is easily followed, so that the endpoints of this test are the malformation rate and the corresponding concentration at which 50% of embryos are malformed (Brannen et al. 2016; Piersma 2011; Schenk et al. 2010).

The accumulation of zinc oxide nanoparticles in mouse embryos induced morphological changes and expression of apoptotic and antioxidant genes in a dose-dependent manner. The maturation process and the number of somites at the end of the exposure were reduced by 80% at the higher dose, and multiple malformations

involved the yolk sac, the heart, the nervous system, the head, structures derived from the branchial arches, and the forelimb (Jung et al. 2015).

While the cited works demonstrated the usefulness and the richness of information of the whole embryo culture, to our knowledge, there are no other works implementing it to study nanoparticles.

3.5.3 *The Multipotent Embryonic Stem Cells*

The embryonic stem cells represent a versatile system to develop different strategies to study in vitro the developmental toxicity. Derived from the inner cells of early blastocyst of different species, they form in vitro a cohesive monolayer that retains the multipotency of the original cells, in the presence of feeder cells and factors that contrast the differentiation. In their absence, the embryonic stem cells start to differentiate and grow as three-dimensional clusters, the embryoid bodies, in which the differentiation spontaneously begins. The embryonic stem cell test approved by the European Centre for the Validation of Alternative Methods included three endpoints, the inhibition of 50% of growth of D3 stem cells line and of mouse embryo fibroblasts (IC50 D3 or other ESCs lines, and IC50 3 T3, respectively), and the inhibition of development of the D3 (ID50). The work of Genschow and co-workers (2004) proposed a classification of chemicals into three classes of hazard (Table 3.3).

The method and the rating scale, validated for bulk chemicals, applied also to the potential embryotoxicity of nanomaterials. Ag, cobalt- or gold-based nanoparticles, with different functionalization, revealed weak or no toxicity (Ahamed et al. 2008; Di Guglielmo et al. 2010). Hereafter, Farcas et al. (2015) studied a panel of metal and metal oxides nanoparticles. Both titanium dioxide nanoparticles, functionalized with hydrophobic or hydrophilic groups, and zinc oxide nanoparticles, coated or uncoated, were strongly embryotoxic (Farcas et al. 2015).

Another method that obtained experimental validation for assessing the embryotoxicity of several nanoparticles was the ToxTracker. Briefly, a panel of six

Table 3.3 Experimental values for rating the toxicity of chemicals in the embryo stem cells. The validation included three endpoints: the concentration inhibiting 50% of growth (IC50) in D3 (embryonic stem cells) and 3 T3 (embryonic fibroblasts) cell lines, and the concentration inhibiting 50% of development (ID50) in D3 cells. (According to Genschow et al. 2004)

Toxicity rating or class	IC50 3 T3	IC50 D3 (ESCs)	ID50
Class I – non-embryotoxic	>100 µg/ml (cut off: 500 µg/ml)	10–1000 µg/ml	>10 µg/ml
Class II – weakly embryotoxic	>100 µg/ml	36–1000 µg/ml	36–1000 µg/ml
Class II – strongly embryotoxic	<40 µg/ml	<10 µg/ml	<9.5 µg/ml

modified mouse embryonic stem cells, each of them expressing a green fluorescent protein linked to a specific stress marker, is grown on a 96 well plate. Oxidative or generic stress, protein unfolding, and DNA damage can be simultaneously monitored within the 24 hours required for the standard test. The validation was reached for metal and metal oxide nanoparticles, while the carbon-based nanoparticles or those present in diesel exhausts did not fulfill the standard. The nanoparticles oxides of zinc, copper, and nickel were weakly embryotoxic ($IC_{50} = 20 \mu\text{g/ml}$), while nickel nanoparticles were strongly embryotoxic ($IC_{50} = 5 \mu\text{g/ml}$). The embryotoxicity was accompanied by markers of oxidative stress and DNA damage. Other nanoparticles including those of titanium dioxide, iron oxide, silver, and cerium dioxide were safe and did not elicit expression of stress or damage markers. Diesel exhaust and multiwalled carbon nanotubes were apparently safe, but the validation was not obtained for these materials (Åkerlund et al. 2018; Karlsson et al. 2014).

In many cases, however, the studies tested only one cell line, and did not calculate the pharmacologic variables IC_{50} and ID_{50} , this last being especially neglected. Some strong embryotoxic compounds represented the exception to the otherwise moderate or absent embryo toxicity of nanoparticles. Among them, the multiwalled carbon nanotubes have limited general toxicity, but more pronounced genotoxicity in mouse embryonic stem cells (Zhu et al. 2007a). Silver and titanium dioxide attracted the main interest because of their diffuse exploitation for commercial products. Cyto- and geno-toxicity were investigated. Silver induced irreversible arrest of cell cycle, reduced expression of multipotency markers and morphological changes. The cytotoxic effects were associated with signs of DNA damage, oxidative stress, documented by hyper expression of specific markers and increased reactive oxygen species. While coating with polysaccharide reduced the cytotoxicity, it seemed not dependent on dissolution (Ahamed et al. 2008; Gao et al. 2017; Karlsson et al. 2014; Park et al. 2011; Rajanahalli et al. 2015). The differentiation of female embryonic stem cells was delayed after exposure to doses generally well tolerated by male stem cells. The altered inactivation of one X chromosome, necessary to start the differentiation, was indicated as the possible underlying mechanism (Zhang et al. 2019).

The titanium dioxide nanoparticles was weakly cytotoxic for the human embryonic stem cells, with an $IC_{50}(24 \text{ h}) = 50 \mu\text{g/ml}$. The exposure induced DNA damage through oxidative stress, apoptosis, altered proteome, and loss of multipotency and of differentiation into cardiomyocytes. In 3 T3 fibroblasts, the compound was not toxic, the adverse effects appeared only at concentration higher than $1000 \mu\text{g/ml}$ and after long-term exposure. The toxicity of other oxide-based nanoparticles, such as those of iron, zinc, nickel, silicon, was absent or weak (Demir et al. 2015; Farcal et al. 2015; Krejčí et al. 2008; Pan et al. 2018; Park et al. 2009).

Gold nanoparticles were safely delivered inside the embryonic stem cells without affecting the viability and proposed as useful probe the embryonic stem cell differentiation. Layers of nanogold organized in the nanoscale provided a safe support for growing and maintaining the multipotency of mouse embryonic stem cells (Lyu et al. 2004; Sathuluri et al. 2011).

The embryonic stem cells, derived from the blastocyst of different species, grow in vitro as a cohesive monolayer that retains the multipotency of the original cells in the presence of feeder cells and factors that contrast the differentiation. In their absence, the embryonic stem cells start to differentiate and grow as three-dimensional clusters, the embryoid bodies, in which the differentiation spontaneously begins. Modified methods allowed the controlled production of embryoid bodies with similar fate, and their development into precursors and specialized tissues, or mini-organs (Dahlmann et al. 2013; Ferguson and Subramanian 2018; Kurosawa 2007; Outten et al. 2012; Xie et al. 2017). These cultures are especially interesting to study the tracking and interplay between cells and nanoparticles in a 3-D space.

A few studies focused on testing the toxicity of nanoparticles on embryoid bodies. Both silver and golden nanoparticles displayed some toxicity, which was strong for the functionalized nanoparticles, the silver citrate and the golden thiolate (Begum et al. 2016; Sathuluri et al. 2011; Senut et al. 2016). Chondroitin sulfate nanoparticles and mesoporous silica were instead highly biocompatible and could promote safe and sustained delivery of growth and differentiation factors in culture, reducing the necessity of frequent inoculation (Garcia-Bennett et al. 2014; Lim et al. 2011). The magnetic nanoparticles, such as superparamagnetic iron oxides, seemed to be also safe; they entered the lysosomal compartment of the cells and gave a significant magnetic signal. In addition, they accelerated the formation of embryoid bodies by attraction of the embryonic stem cells in a variable magnetic field and could induce the mesodermal cardiac differentiation through magnetically induced mechanical distortion (Du et al. 2017; Nejadnik et al. 2012; Parsa et al. 2015).

Special interest developed on the neurotoxicity of nanoparticles on the embryonic stem cell-derived and embryoid bodies with neural differentiation. All the nanoparticles tested, such as polyethylene, iron oxide, double-layered magnesium and aluminum hydroxides, and silver, were neurotoxic. The neural differentiation and the associated gene expression were reduced. In some cases, death, apoptosis, and oxidative stress appeared around 100 µg/ml (Hoelting et al. 2013; Oh et al. 2016; Rostami et al. 2015; Wu et al. 2015).

In conclusion, the embryonic toxicity of nanoparticles, evaluated with the embryonic stem cell test method and its variants, is generally moderate, the only exception being functionalized nanoparticles and certain metal or metal oxides.

3.5.4 Zebrafish Embryo Test

The Zebrafish Embryo Test is a variant of the Fish Embryo Test, planned to test the environmental pollution and including also the medaka and the fathead minnow as model species. The zebrafish embryonic test is highly standardized and validated to test the embryotoxicity; its predictivity is demonstrated, and the guidelines are available as published papers or online documents (Beekhuijzen et al. 2015; Brannen et al. 2016; Ducharme et al. 2013; Dumitrescu et al. 2019; Hamm et al. 2019; Haque

and Ward 2018; Jia et al. 2019; OECD 2013; Piersma 2011) (Table 5.1). Recently, the zebrafish embryonic test was also standardized for testing metallic nanomaterials (Pecoraro et al. 2017) and to assess the biodistribution of polystyrene nanoparticles (nanoplastics) in the developing embryo (Lee et al. 2019; van Pomerén et al. 2017a). The test is versatile, performing well to assess the toxicity and the embryotoxicity in pharmacology as in ecotoxicology, requires small quantities of nanomaterials, is reproducible, predictive, highly standardized, and easy to perform also in automated systems. Herein, we present a synthetic overview of the results.

Among the alternative methods for embryotoxicity, the zebrafish embryonic test was the most used to test the carbon-based nanomaterials. The exposure to buckminsterfullerene, such as C60, C70, and C98, induced excess of mortality and heart and caudal fin malformations at doses lower than 1.5 µg/ml. concentration inducing 50% lethality in the sample was very low (LC50 = 0.13 µg/ml). The surface hydroxylation dramatically reduced the toxicity, as did the quenching of oxidative stress with glutathione added to the medium and exposure under dim light (Isaacson et al. 2017; Usenko et al. 2007, 2008; Zhu et al. 2007b). Carbon black was safe, at least in experimental conditions adopted in the work of Cheng et al. (2007). The toxicity of carbon nanotubes, single-, di-, or multi-walled, was moderate. In embryos exposed from the 2-cell stage, they were distributed in all the cells of the blastoderm, and were excluded from the yolk cells. If injected into the embryonic circulation, they persisted after hatching, the complete clearance requiring 96 hours; however, the germ cells of the embryo were not damaged, and the adult developed normal gametes (Cheng et al. 2009). Instead, aggregates in unstable solution were larger than the pores of the chorion; the nanotubes being therefore unable to reach the embryo, the observed toxicity was attributed to the ions of heavy metals present in a residual of the production (Cheng et al. 2007). Pegylation did not improve the stability of single-walled carbon nanotubes, which retained a certain degree of toxicity without reaching the 50% of mortality or of malformation (Cordeiro et al. 2018). Delayed hatching and growth, pericardial edema, and malformations of the caudal fin appeared after exposure to at least 60 µg/ml. Their toxicity increased if the length of the tubes was reduced by prolonged sonication (Asharani et al. 2008; Cheng et al. 2007; Cheng and Cheng 2012; Liu et al. 2014). A more detailed investigation demonstrated that the nanosheets enveloped the chorion, blocked the pores, and induced severe hypoxia. Consequently, dose-dependent mitochondrial damage, apoptosis, delayed hatching, and multiple malformations followed (Chen et al. 2015). Some carbon-based nanoparticles were intended as fluorescent probes for imaging, such as graphene and carbon quantum dots, and nanodiamonds. All performed well in the zebrafish embryo, with null or low toxicity, according to the authors (Lin et al. 2016; Wang et al. 2015; Xu et al. 2014b). Moreover, *in vivo* studies suggested that the lethal and theratogenic effects in mammals were much more severe than those induced in the zebrafish (Ema et al. 2016).

The zebrafish embryonic test performed well to assess the toxicity of metal nanoparticles. The exposure to gold was safe for spherical nanoparticles, while nanorods had a dose-dependent lethality (LC50 < 0.01 nM). Coating with organic polymers enhanced the bioavailability and the toxicity, with some exception, such

as the nanorods coated with polystyrene or polyallamine (Asharani et al. 2011; Böhme et al. 2015; Kim et al. 2013; Pan et al. 2013; Patibandla et al. 2018; Ramachandran et al. 2017). Platinum nanoparticles are important environmental pollutants that caused hatching delay, low heart rate, deformed spine, and anomalous behavior of larvae after exposure during the embryonic age (Asharani et al. 2011).

The silver nanoparticles, widely present in commercial products, had a special place in studies with the zebrafish embryonic test and represented more than 30% of the study here considered. They reached the embryo crossing the chorion membrane through the pores, with a passive mechanism sustained by Brownian movements. Their toxicity involved all three endpoints, which are mortality, delayed hatching, caudal fin malformation and low heart rate; 100% mortality followed the exposure to 20 nM (Lee et al. 2007, 2012a, b, 2013b), while the ID50 for pericardial edema was ca. 2 µg/ml, higher for axial malformations (Eryilmaz et al. 2018). Similar results were also obtained in fish embryo test utilizing the medaka (Wu and Zhou 2012). The silver embryotoxicity was higher than that of gold, platinum, cadmium/selenium, and zinc oxide (Asharani et al. 2011; Lacave et al. 2016). Several factors influenced the toxicity of silver nanoparticles, such as the salinity of the water, the shape of nanoparticles, their functionalization at the surface, the embryonic stage at which the exposure started, and the presence of the chorion. Salt water was safer than freshwater (Heinlaan et al. 2016), and nanoparticles shaped as plates were more toxic than spheres or rods; moreover, the side effects of spheres totally depended on the dissolved ions, while that of plates and rods, coated with polyvinylpyrrolidone, was due to the nanoparticles (van Pomeroy et al. 2017b). The diffusion through the chorion was independent of the stage at which the embryo was exposed, while the toxicity differed. The early gastrula and the hatched larvae were resistant, while cardiac abnormalities developed after exposure at early and late segmentation stage (0.02 nM). Then, exposure at the cleavage, pre-organogenesis stage, caused multiple abnormalities of the spine, yolk sac edema, and acephaly (Lee et al. 2013a). The functionalization at the surface with organic polymers did not alter the passive crossing of the chorion (Lee et al. 2013b), while the toxicity was differently affected. In general, neutral coating was safer, and negatively charged more hazardous, but this is not a rule as pointed out by the various and rich literatures in this field (Kim et al. 2013; Kim and Tanguay 2014; Lee et al. 2013b; Orbea et al. 2017; Park et al. 2013; Powers et al. 2011; Truong et al. 2012). Notwithstanding their proved toxicity, a study reported the exploitation of Ag nanoparticles to imaging the segmentation of the zebrafish embryo. The nanoparticles were photostable, diluted in solution that remained stable for months, and had plasmonic properties. Chorion revealed different zones of viscosity, and counter-clockwise flow patterns (Nallathamby et al. 2008).

The health hazard of titanium dioxide nanoparticles, present in the formulation of many commercial products, became also threatening as environmental pollutants. Recently, a study tested the interaction of nanoparticles with a polymer used for water remediation: the zebrafish embryo was grown in the presence of the Nafion polymer combined with various fillers, such as anatase-type TiO₂, without associated lethal or sublethal effects (Pecoraro et al. 2018). The size of non-functionalized

TiO₂ nanoparticles played a role in toxicity, the larger being safer than smaller, with a value of LC50(120 hpf) = 23.4 µg/ml for nanoparticles sized 6 nm (Kim et al. 2014). The shape of nanoparticles, instead, did not play a major role: embryos were safely exposed to spheres and plates, and only bipyramidal nanoparticles induced some increase of malformation, no more than 20% at the highest concentration used (van Pomerén et al. 2017b). The titanium dioxide nanoparticles were safe at concentration as high as 1000 µg/ml, also in another alternative model organism, the amphibian, *Xenopus laevis* (Nations et al. 2011). The most important characteristic of titanium dioxide is its photo-activity: under direct sunlight or UV irradiation, the production of reactive oxygen species greatly increases, with dramatic changes of embryotoxicity. The yolk-sac larvae were the most sensitive stage to the phototoxicity of titanium dioxide nanoparticles, while embryos, free-swimming larvae, and juvenile were resistant (Ma and Diamond 2013). The effect of UV irradiation enhanced the malformation rate of plate-shaped nanoparticles (ID50 = 0.015 µg/ml), and that of spheres and bipyramids remained unchanged. The mortality rate under irradiation was increased in all differently shaped nanoparticles; however, LC50 was never reached (van Pomerén et al. 2017b). The weak or absent toxicity observed in zebrafish embryonic test with photo-inactivated TiO₂ nanoparticles doped in sulfur- or sulfur and fluorine is a countercheck of the fundamental role of irradiation (Pathakoti et al. 2013). As a general remark, the titanium dioxide nanoparticles are weak embryotoxic in itself, but can represent a threat for many species under sunlight or UV irradiation.

The zinc oxide nanoparticles are also widely diffused. Their toxicity included reduced hatching and increased heart rate, possibly due to hypoxia, in zebrafish embryos exposed to 10–120 µg/ml. The toxic mechanisms included the oxidative stress and apoptosis, with reduced potential of the mitochondrial membrane and altered expression of genes related to both processes (Zhao et al. 2016). The embryo toxicity of zinc oxide nanoparticles increased the theratogenicity of an environmental pollutant, the perfluorooctane sulfonate (Du et al. 2016). However, the mortality rate and the prevalence of severe malformations after exposure to zinc oxide were not reported in zebrafish embryonic test. The results obtained in *Xenopus laevis*, in which ID50 (96 h) = 10.3 µg/ml and 89% of gut malformation were recorded (Nations et al. 2011), suggest species-specific sensitivity to these nanoparticles. Attempts to reduce the dissolution from nanoparticles included doping in silver, which reduced the hatching failure. The engineered nanoparticles of zinc oxide in a shell of gadolinium oxide were intended as a probe for imaging. They anticipated the hatching at 72 hpf, but induced multiple malformations, dose-dependent in incidence and severity (Woźniak et al. 2017).

Other metal- and metal oxide-based nanoparticles have been tested with the zebrafish embryonic test. Copper-based nanoparticles were weakly toxic, with LC50 greater than 1000 µg/ml in standard medium and after 96 hour of exposure (Nations et al. 2011), and over 100 µg/ml in natural freshwater (Heinlaan et al. 2016). Another study reported lower values, LC50 = 10 µM and ID50 = 0.5 µM for delayed hatching (Thit et al. 2017). The effect of nanoparticles and ions were comparable, the dissolution low. Copper nanoparticles seemed to be more toxic to

the zebrafish embryo. The mortality increased over the concentration over 0.1 µg/ml, and delayed hatching and malformation appeared within 0.01 and 0.05 µg/ml. The highest concentrations of nanoparticles were more toxic than equivalent doses of ions (Bai et al. 2010). The malformation of the posterior swim bladder was associated to downregulation of *Wnt*-signaling, identified by the authors as the pathogenetic mechanism (Xu et al. 2017).

The embryonic toxicity of iron oxide nanoparticles was possibly species-specific. In zebrafish, toxicity in terms of mortality, hatching delay, and malformation appeared at concentration of ca 10 µg/ml (Zhu et al. 2012). In *Xenopus laevis*, the toxic doses were much higher, with LC50 > 1000 µg/ml (Nations et al. 2011).

Other nanoparticles, tested with zebrafish embryonic test, are the quantum dots, powerful fluorescent probes whose usefulness in imaging living organisms is limited by their toxicity. The cadmium/selenium quantum dots in a shell of zinc confirmed their toxicity for the zebrafish embryo, which was greater for “aged” quantum dots, obtained simulating the process occurring in the soil, which causes the erosion of the shell of zinc and the dissolution of toxic species. A similar degree of toxicity was retained by the cadmium/selenium quantum dots; further data confirm the role played by dissolution in causing embryonic toxicity (Lacave et al. 2016; Wicinski et al. 2013).

Nanoparticles based on organic polymers, lipids, and non-metal oxides are usually engineered for special purposes, such as drug delivery, transfection, labeling of cells and tissues, obtaining advanced materials for bioremediation. Non-metal oxides, such as Cerium oxide and nanoporous silica functionalized with amine groups, were not toxic (Matos et al. 2016; Van Hoecke et al. 2009). Quinoxaline-based polymer dots are powerful probes for in vivo imaging and cellular labeling without the need of specific targeting. They were not toxic when tested with the zebrafish embryonic test (Liu et al. 2015). Other organic nanoparticles were conceived for drug transport and delivery, such as polyphenylene and polyamidoamine dendrimer, liposomes and the nanocapsules. The safer among them, as tested with the zebrafish embryonic test, were the polyphenylene dendrimers (Stangenberg et al. 2015), the phosphatidylcholine liposomes (Sieber et al. 2017) and the nanocapsules with a double shell of hyaluronic acid and protamine (Teijeiro-Valiño et al. 2017). All these appeared to be suitable platforms for drug and molecules release, and the nanocapsules were also tested for their ability to cross the chorion, as a representative biological barrier. They were able to cross the chorion and persist in circulation. On the contrary, the polyamidoamine dendrimers displayed lethal and sublethal embryo toxicity, higher for positively charged dendrimers (Calienni et al. 2017). The liposomes with positive charges at the surface had also severe embryotoxicity (Sieber et al. 2017).

3.6 Conclusions

The potential applications of nanomaterials in vertebrate controlled reproduction, pre-natal cure, and regenerative medicine seem to be high, and require adequate assessment of safety.

The advancement of artificial insemination techniques requires techniques for sperm sorting, to select the gametes with higher fertilizing performance. In the case of livestock, obtaining a sufficient amount of sperm bearing the X chromosome permits the generation of economically valuable females, avoiding the need of male castration. Techniques for sperm sorting became available decades ago; however, the yield of traditional methods is poor, especially for livestock in which, like in the swine, the selection of male gametes begins in the vagina, not in the uterine channel (Rath et al. 2015). Several types of nanoparticles efficiently sort the sperm, with null or minimal reproductive toxicity (Barkalina et al. 2016; Odhiambo et al. 2014; Rath et al. 2015). Other nanoparticles improve the growth and differentiation of the early embryo, cross the placenta (Grafnmueller et al. 2013, 2015a, 2015b) and deliver drugs to the zygote (Ali et al. 2013; Lopalco et al. 2015; Menjoge et al. 2011; Remião et al. 2016), or do not cross the placenta and delivery the drugs to the mother without exposing the fetus (Menezes et al. 2011).

Transfection without a viral capsid is another interesting field for the theragnostics, and some nanoparticles can safely do it in gametes and embryos (Jin et al. 2016; Kim et al. 2010; Munk et al. 2016; Park et al. 2017; Remião et al. 2017). The differentiation of stem cells into precursor and specialized cells is a promising field for the advancement of regenerative medicine (Grande et al. 2017; Kingham and Oreffo 2013; Leung et al. 2013; Ling et al. 2017; Shtrichman et al. 2014; Shahbazi et al. 2011). Cardiac repair, (Ban et al. 2014), construction of vascular tissues (Hu et al. 2012), neuronal regeneration (Hackelberg et al. 2017; Yang et al. 2014), and osteogenic differentiation (Smith et al. 2010) are only few examples of the great potential of nanomaterials in theragnostics.

As a consequence of all these attractive perspectives, the assessment of the reproductive safety of nanomaterials is necessary and requires appropriate methods and tests. The traditional tests *in vivo* are costly and time-expensive; therefore, the alternative methods represent a real step in the field. This review tried to draw a rational scheme of the numerous studies on the argument. The overall landscape reveals an informative body of knowledge, with great potential for newer exploitations of nanomaterials in husbandry, veterinary, and human medicine. The reproductive toxicity of many nanomaterials is moderate and acceptable. Waiting for well-defined recommendations of the regulatory agencies, still lacking, the panel of alternative tests for reproductive toxicity proposed for traditional chemicals seems to be adequate for the study of nanomaterials, eventually with some modifications *ad hoc*. Some reviews that discuss the reproductive safety of nanomaterials and underline the necessity of studies better focused on the characterization and dosing of the potential toxic compounds, seem authoritative (Ema et al. 2016; Liu et al. 2016a). Some frailty affecting the bodies of studies here considered is the lacking, in several

cases, of the comparison of the effects of nanomaterials and of the chemical species entering the system after their dissolution. Again, it is important that all studies concerning the reproductive safety/toxicity of nanomaterials should include variables appropriate to rate the toxicity. Among these variable, we recall at least the IC₅₀ (or ID₅₀), and the LC₅₀, that are the concentrations lethal for the 50% of the observed biological system, or inhibiting the same percentage of growth in cultured cells. IC₅₀ refers to concentration, ID₅₀ to dose.

To conclude, the alternative methods *in vitro* are essential for the assessment of the reproductive toxicity of nanomaterials, being predictive and efficiently reducing the burden of traditional investigation of the reproductive safety, under the point of view of the R3 concept, that is, reduction, refinement, and replacement of the necessary tests in mammals.

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Chapter 4

Nano-toxicity to Microbes: Potential Implications of Nanomaterials on Microbial Activity



Hemraj Chhipa

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Abstract The increasing concentration of engineered nanomaterials and their accumulation in the environment is the major concern for toxicity to soil microbial community. The engineered nanomaterials have shown positive and negative effect on microbes, microbial physiology, and their enzymatic activities, which play a significant role in various biogeochemical cycles to maintain ecosystem. The disorganization of microbial community structure affects the soil fertility and ecosystem, which involved in disintegration of complex substrates, remediation of pollutants, and plant growth. Various metal nanoparticles and carbon-based nanomaterial have shown their role in induction or suppression of various microbial enzymatic

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V. Kumar et al. (eds.), *Nanotoxicology and Nanoecotoxicology Vol. 1*, Environmental Chemistry for a Sustainable World 59, https://doi.org/10.1007/978-3-030-63241-0_4

activities like dehydrogenase, phosphates, urease, nitrogen fixation, and ammonia oxidation. Therefore, we reviewed the effect of different types of engineered nanomaterials such as silver, zinc oxide, copper oxide, titanium oxide, iron, and carbon-based nanomaterial on the microbial community structure, which is needed for the harmonization of soil and water ecosystem. Silver, copper, and carbon-based nanomaterial have been studied broadly in terms of toxicity to microbial communities. The toxicity level of various nanomaterials depends on nanomaterial concentration and structure of exposed microbial community. The mechanism of nanomaterial toxicity to microbial enzymatic activities, role of nanomaterials in reactive oxygen species generation, and their impact on microbial reduction have been discussed in the chapter.

Keywords Microbial community · Nanomaterial · Nano-toxicity · ROS · Enzyme

4.1 Introduction

Microbes are the most important component of ecosystem, which play a significant role in different biogeochemical cycles, climate regulation, and plant productivity. The diversity of microbial community disturbed by changes in the surrounding environment such as increasing in organic or inorganic components, temperature, pH and water, which effect the expression of enzyme and their activity.

In the current decade, the application of metal nanoparticles is expanding in agriculture, medicine, food industry, electrical and electronics, and nanotechnology-based consumer products are increasing. Simultaneously, the demand of nanomaterial production increased with time. It was predicted that about 58,000 tons of engineered nanomaterials have been produced by 2020 (Maynard et al. 2006). The synthesized nanomaterials are released into environment from various industries or through anthropogenic activities, for example, zinc oxide (ZnO) and titanium oxide (TiO₂) nanoparticles have been released indirectly through the use of ZnO- and TiO₂-containing sunscreens and cosmetic products. Similarly, silver nanoparticles are released from washing textile industries, and TiO₂ nanoparticles are released from paint industries. Uses of nanoparticle-containing fertilizer and pesticides also contaminate the soil and water bodies (Osmond and McCall 2010; Kaegi et al. 2010; Lorenz et al. 2012; Nowack et al. 2012).

The release of excess nanomaterial in the environment affects the different components of the ecosystem, and microbes are one of them. The shape, size, surface chemistry of nanoparticles, and chemical composition are the major characteristics of metal nanoparticles which are responsible for biological interactions (Sinha and Khare 2013). Characteristics such as nanoscale size and high surface to volume ratio of nanomaterials make them highly active on the basis of their ionization nature. The released nanomaterials interact with the microbial communities residing in different habitats such as soil, water, and air and show toxicity to them. Different types of nanomaterials have been reported, such as silver nanoparticles

(Ag NP), carbon nanotubes (CNT), copper nanoparticles (CuO), iron-based nanoparticles (Fe NP), graphene-based nanomaterials, zinc nanoparticles (ZnO), and titanium oxide nanoparticles (TiO₂ NP), which showed nano-toxicity to microbial communities at various concentration levels (Tong et al. 2007; Johansen et al. 2008; Ge et al. 2011). The toxicity of nanoparticles also depends on the type of microbes, for example, Gram-negative microbes reported more susceptibility due to lesser peptidoglycan layer in the outer membrane in comparison to Gram-positive bacteria.

In the current chapter, we review the effect of various nanoparticles on microbial community and their enzyme activity and mechanism of nano-toxicity to microbes. The diversified impact of nanomaterials on microbial community and their enzymatic activity still needs more research to understand the effecting concentration threshold of nanomaterials and their role on the expression of enzymes at the community level. Further, the interaction of nanomaterials at complex microbial community is required to understand the nano-toxic effect on different parts of biogeochemical cycles.

4.2 Nanomaterials in Environment

Nanomaterials enter in soil through the use of sewage sludge as fertilizer and spray of nanopesticides as plant protectants (Larue et al. 2014). The use of nanoparticles as antibacterial agent in medical industry, paint industry, food industry, catalysis, water purification systems, and building materials increase their concentration in environment (Aitken et al. 2006; Sharma 2009; Hossain et al. 2014). Collins et al. (2012) studied the mobility of copper (Cu) and zinc oxide (ZnO) nanoparticles in open pot system and reported that high organic content in soil provides the organic coating on Cu and ZnO, which support the mobility of nanoparticles in soil. The mobility of the nanoparticles depends on ionic strength, pH of the medium, and organic matter. The copper nanoparticles are found least mobile in comparison to copper oxide, iron oxide, titanium oxide, and zinc oxide nanoparticles. The dissolution and transformation of nanoparticles are also obtained with time in environment which affect the concentration of nanoparticles.

Nanoparticles interact with dissolved organic matter, multivariate cations or anions and natural colloids, which affect the stability of nanoparticles and their aging process. The coating of nanoparticles also plays a significant role in their stability, which decides the interaction of nanoparticles with the surrounding environment and their aggregation. Erhayem and Sohn (2014) found that adsorption constants of organic material to titanium oxide nanoparticles decreased in the order humic acid (HA) > natural organic matter > fulvic acid (FA), which showed with different degrees of stabilization. Tween 80-coated silver nanoparticles showed more stability in comparison to citrate-coated silver nanoparticles in freshwater medium, but still there are no specific determinants that can predict which conditions like coating, natural organic matter, surrounding cations, pH, engineered

inorganic nanoparticles characteristic, and initial capping agent provide the stability in soil and water systems (Schaumann et al. 2015). Presence of humic substance and polysaccharides in natural organic matter also play a role in molecular bridge formation between particles (Liu et al. 2010).

4.3 Interaction of Nanomaterial with Microbial Communities

Microbes are an important component of the ecosystem and play a significant role in ecological, hydrological, and geological cycles. Change in microbial community structure directly affects the environmental quality and human health. Nanomaterial interacts with microbes and disrupts their cell membrane or interferes with cell signaling, resulting in microbial disruption or induction of cell growth (Shahverdi et al. 2007). Distraction of microbial community structure affects the bottom of food chain which impacted the soil health system in long term.

Matsumura et al. (2003) reported the possible reasons for toxicity of nanomaterial in microbes including interactions of nanomaterial with –SH group in respiratory enzymes prevent respiration, produce proton leakage by transport protein binding, and removal of intracellular phosphate ions hinders phosphate inhibition and pyrimidine dimerization by DNA binding. Galindo et al. (2013) studied the effect of different nanoparticles against white-rot fungi species *Lentinus sajor caju*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor* at lab scale. They found that metal nanoparticle (silver nanoparticles), Quantum dots (zinc sulfite), metal oxide nanoparticles (Mo/NaO), and organic nanoparticles (SDS/DDAB) significantly inhibited the growth of fungi with effects on the mycelium chemical composition. It was observed that nanoparticle toxicity was not size-dependent because nanoparticles get aggregated in milli-Q water and increase in size, but the toxicity was found due to dissociation of ions from respective metal nanoparticles. The quantum dots showed maximum toxicity, which has been reported as a cause of plasma membrane damage, mitochondrial membrane damage, and DNA fragmentation, which leads to the cell death (Hardman 2006).

Engineering nanomaterial (ENP) changes the physical properties of soil by changing nutrient availability and increases bioavailability of contamination and indirectly affected the soil microbial community.

4.4 Effect of Nanomaterials on Soil Microbial Flora

Soil microbial flora plays a huge role in soil fertility; it improves mobilization of metals for plant growth. The health of soil is directly influenced by the presence of biotic parameters (Doran and Zeiss 2000). Soil contains large numbers of organisms

such as bacteria, fungi, nematode, protozoa, and arthropods, which play a significant role in the sustainable food chain of the local ecosystem.

The increasing number of nanomaterial-based consumer products enhances the amount of nanomaterials in soil. The accumulated nanomaterials, interacted with different soil microbes and affected their activities (Table 4.1). The interaction of different nanomaterials with different soil microbial communities influences microbial flora in both ways. Some nanomaterials induce the abundance of soil microbes and some destroy the community structure. The positive and negative impact of nanomaterial on microbial flora depends on the concentration and dissolution rate of nanomaterials present in the soil.

Johansen et al. (2008) reported that carbon nanomaterial does not influence the soil microbial community and it can be a potential tool for environmental application, but they found a little change in the number of fast-growing bacteria. Reduction in the number of fast-growing bacteria affected the protozoan community because they feed on the bacteria. The chemical nature of nanoparticles also plays a significant role in their toxic nature. Moll et al. (2016) reported that multi-walled carbon nanomaterial more influenced the symbiotic microbial activity in comparison to titanium oxide and cerium oxide nanoparticles. The toxicity of nanoparticles on monoculture was found due to disorganization of cellular membrane, DNA damage, generation of reactive oxygen species, and photocatalytic oxidation of nanoparticles. In soil, nanoparticles activity was affected by agglomeration, adsorption, desorption, dissolution, and migration by different soil properties like pH, ionic strength, clay content, soil moisture, and organic matter content. The above-reported factors changed the concentration of nanomaterials and showed variability in terms of toxicity toward microbial community (Brayner et al. 2006; Choi and Hu 2008; Gou et al. 2010).

The toxicity of nanoparticle in soil depends on the dose and soil type. In some cases, low dose showed enhancement of microbial biomass while in contrast some cases reduction in microbial biomass measured (Pan and Xing 2012). Wu et al. (2010) reported that metal nanoparticle can be aggregated and formed large agglomerate which reduce the nanoparticle toxicity.

Soil pH is one of the important parameters, which influences the toxicity of the nanomaterial by playing a significant role in their dissolution, mobility, and migration to soil microbial community and plants (Heggelund et al. 2014, Waalewijn-Kool et al. 2013). Schlich and Hund-Rinke (2015) studied the effect of soil types on nano-toxicity to microorganism and found that in acidic soil, silver nanoparticle toxicity was more in comparison to alkaline soil. They observed that soil pH influences the release of ions via dissolution of metals. Previously, Waalewijn-Kool et al. (2013) also found zinc oxide toxicity in their study and stated that metallic nanoparticle toxicity was dependent on soil pH and soil acidity increased the toxicity.

The concentration of nanoparticles and exposure time also affect the toxicity. Collins et al. (2012) measured no significant change in the community structure of *Rhizobiales* at 550 mg by copper and zinc oxide nanoparticles after 160 days of exposure in culture-independent FAME analysis. But they found little change in order *Flavobacteriales* due to copper nanoparticles. They suggested that microbial

Table 4.1 Effect of nanomaterials on soil microbial community: Different types of nanomaterials showed positive and negative impact on microbial community which affect the soil activity and fertility. The table listed different types of microbial communities present in soil and their response against nanoparticle exposure

Nanomaterial	Microbial community	Concentration	Impact	Reference
Ag NP	<i>Proteobacteria</i> , <i>Acidobacteria</i> , and <i>Verrucomicrobia</i>		Increased Proteobacteria; reduction in Acidobacteria and Verrucomicrobia	McGee et al. (2017)
Ag NP	<i>Rhizobium leguminosarum</i> and <i>Glomus aggregatum</i>	800 ppm	Slowdown in nodulation, nitrogenase activity, and colonization	Abd-Alla et al. (2016)
Ag NP	Mycorrhizal community	2.5 mg.kg ⁻¹	Decrease diversity of mycorrhizal fungi	Cao et al. (2017)
Ag NP	Ammonia-oxidizing bacteria		Reduction in microbial abundance	Schlich and Hund-Rinke (2015)
Ag, ZnO, and TiO ₂ NP	<i>Sinorhizobium meliloti</i>		Reduction in nodulation frequency	Judy et al. (2015)
Au-NP, CdSe, and ZnS	<i>Lentinus sajor caju</i> , <i>Phanerochaete chrysosporium</i> , <i>Pleurotus ostreatus</i> , and <i>Trametes versicolor</i>		Inhibition of fungal growth	Galindo et al. (2013)
CNM	Bacteria population		Reduction in fast growing bacteria	Johansen et al. (2008)
CNM	Microbial community		Reduction in microbial respiration	Goyal et al. (2010) and Tong et al. (2012)
MWCNT	Arbuscular mycorrhiza	3000 ppm	Increment in colonization	Moll et al. (2016)
MWCNT	<i>Waddlia</i> , <i>Holophaga</i> , <i>Derxia</i> , and <i>Opitutus</i>	10,000 mg. kg ⁻¹	Reduction in size of microbial community	Shrestha et al. (2013)
MWCNT	Soil microbial community	50 and 200 µg/mL	Abundances of Bacteroidetes and Firmicutes increased, and Proteobacteria and Verrucomicrobia decreased	Khodakovskaya et al. (2013)

(continued)

Table 4.1 (continued)

Nanomaterial	Microbial community	Concentration	Impact	Reference
SWCNT	Microbial community	0.03–1 mg. g ⁻¹	Reduction in microbial biomass	Jin et al. (2014)
SWCNT	Soil microbial community	1000 µg.g ⁻¹	Lowered microbial biomass	Jin et al. (2013)
CeO ₂ NP	<i>Ensifer</i> , <i>Rhodospirillaceae</i> , <i>Clostridium</i> , and <i>Azotobacter</i>	0.1–0.5 ppm	Number decreased	Ge et al. (2014)
CeO ₂ , TiO ₂ , and ZnO NP	Potassium and phosphate reduction bacteria	1 mg	Reduction in abundance	Chai et al. (2015)
Cu NP	<i>Flavobacteriales</i>		Significant change in community structure	Collins et al. (2012)
Cu NP	<i>Bacillus subtilis</i>	60 µg	Inhibition	Ingle et al. (2014)
Fe ₂ O ₃ and Fe ₃ O ₄ NP	Bacteria population		Positive impact on bacterial population	He et al. (2011)
TiO ₂ NP	Ammonia-oxidizing archaea and bacteria, <i>Nitrobacter</i>	< 1 mg.kg ⁻¹	40% reduction in number	Simonin et al. (2017)
ZVF NP	β and γ-proteobacteria	34,000 ppm	Decline in number	Fajardo et al. (2012)
ZVF NP	Arbuscular mycorrhizal fungi and Gram-negative bacteria		Negative impact on microbial abundance	Pawlett et al. (2013)
ZnO NP	<i>Candida albicans</i>	1 ppm	95% growth inhibition	Lipovsky et al. (2011)
ZnO NP	<i>Skeletonema marioni</i> , <i>Thalassiosira</i> <i>pseudonana</i> , <i>Dunaliella</i> <i>tertiolecta</i> , and <i>Isochrysis galbana</i>	1 ppm	50–75% decrease in growth	Miller et al. (2010)
ZnO and TiO ₂ NP	<i>Bradyrhizobiaceae</i> , <i>Geodermatophilaceae</i> , <i>Methylobacteriaceae</i> , <i>Micromonosporaceae</i> <i>Rhodospirillaceae</i> , <i>Actinoplanes</i> , <i>Balneimonas</i> , <i>Blastococcus</i> , <i>Bradyrhizobium</i> , and <i>Skermanella</i>	0.05–0.5 ppm	Decline in microbial abundance	Ge et al. (2012)

(continued)

Table 4.1 (continued)

Nanomaterial	Microbial community	Concentration	Impact	Reference
Silver-graphene oxide nanocomposite	<i>Acidobacteria</i> , <i>Firmicutes</i> , and <i>Cyanobacteria</i>	0.1–1 mg g ⁻¹	Negatively affect soil microbial activity	Kim et al. (2018)
MoO ₃ NP NiO NP Li ₂ O NP	Archaea, Bacteria, and Eukarya	2–173 µg 11–1018 µg 4–474 µg	Microbial community structures shifted	Avila-Arias et al. (2019)

CNM carbon nanomaterial, MWCNT multi-walled carbon nanotube, SWCNT single-walled carbon nanotube, ZVF Zero valent iron nanoparticles

strains showed different mechanisms of susceptibility toward different types of nanoparticles. Ge et al. (2012) reported that nano zinc oxide and titanium oxide showed dose-dependent toxicity in soil bacterial community. They observed that *Bradyrhizobium*, *Bradyrhizobiaceae*, *Rhizobiales*, and *Methylobacteriaceae* taxa were declined with increasing concentration of zinc oxide and titanium oxide nanoparticles in the range of 0.05–0.5 ppm, while *Sphingomonadaceae*, *Streptomyetaceae*, and *Streptomyces* were found abundant after nanomaterial exposure. They revealed the changes in community structure with a decreasing number of *Bradyrhizobiaceae*, *Geodermatophilaceae*, *Methylobacteriaceae*, *Micromonosporaceae*, *Rhodospirillaceae*, *Actinoplanes*, *Balneimonas*, *Blastococcus*, *Bradyrhizobium*, and *Skermanella* after exposure of zinc oxide and titanium oxide nanoparticles and found both nanoparticles also reduced the microbial biomass.

In another study, Fajardo et al. (2012) studied the impact of zero valent iron (ZVF) nanoparticles in sandy loam clay and found that nanoparticles showed a mixed effect on microbial community. The abundance of α -proteobacteria and archaea was increased while β and γ -proteobacteria decreased at 34000 ppm concentration. Similarly, Pawlett et al. (2013) found a change in microbial community structure after treatment with zero valent Fe nanoparticles in sandy, loam, and clay soil. They reported negative impact on arbuscular mycorrhizal fungi and Gram-negative bacteria. The effect of Ag nanoparticles on symbiotic microorganism was studied by Abd-Alla et al. (2016). They reported that at 800 ppm concentration, silver nanoparticle inhibited the growth of *Rhizobium leguminosarum* and *Glomus aggregatum* in soil. They measured slowdown in nodulation, nitrogenase activity, colonization of mycorrhizal fungi, and deterioration of intracellular cytoplasmic component.

Cerium oxide nanoparticles also altered microbial community composition at 0.1 ppm concentration, while zinc oxide altered them at 0.5 ppm. The bacterial community *Rhizobium* and *Sphingomonas* was increased and *Ensifer*, *Rhodospirillaceae*, *Clostridium*, and *Azotobacter* were decreased (Ge et al. 2018). Similarly, Judy et al. (2015) also reported that the presence of silver, zinc oxide, and titanium oxide nanoparticles reduced the nodulation frequency of symbiotic bacteria *Sinorhizobium meliloti* in *Medicago truncatula* and shifted the microbial community composition. Different metal oxides such as cerium oxide, silicon oxide,

zinc oxide, and titanium oxide at 1 mg concentration showed both positive and negative response on microbial community. It was observed that zinc oxide and cerium oxide reduced the abundance of potassium and phosphorus reduction bacteria and hindered their enzymatic activity and silicon oxide increased their activity (Chai et al. 2015).

The carbon nanomaterial did not affect soil microbial activity at short-term experiment but changed C-14 glucose incorporation into bacterial biomass at 1–1000 mg.kg⁻¹ concentration (Oyelami and Semple 2015). Recently, Ge et al. (2018) studied the effect of carbon nanomaterial on soil microbial community and observed the alternation of microbial community composition related to carbon (C), nitrogen (N), and sulfur (S) biological cycles.

Chen et al. (2017) found low toxicity of silver, zinc, and titanium nanoparticles to soil microbial community and measured abundance in Gram-negative and anaerobic bacteria at low concentration of engineered nanoparticle (ENP). Similarly, Simonin et al. (2017) also found the toxic effect of titanium oxide on ammonia-oxidizing archaea and bacteria, *Nitrobacter* and *Nitrospira*, which play a significant role in nitrite oxidation. They found about 40% reduction in ammonia-oxidizing archaea, *Nitrobacter* and ammonia-oxidizing bacteria after 90 days of exposure at <1 mg.kg⁻¹ concentration.

Nature of metal in nanoparticle also affects the microbial community at the same concentration level. Asadishad et al. (2018) studied the effect of silver, copper oxide, zinc oxide, and titanium oxide at 1–100 mg.kg⁻¹ concentration on microbial community and enzyme activity and found that after 30 days of exposure zinc oxide and copper oxide did not affect or increase the soil enzyme activity but in contrast, silver inhibited enzyme activity at high concentration, while titanium oxide did not change significantly. Cao et al. (2017) found that silver nanoparticles influenced mycorrhizal community in maize plant at 2.5 mg.kg⁻¹ concentration. They reported that anti-oxidation activity of silver decreases diversity of mycorrhizal fungi, resulted in decrease in colonization rate of root mycorrhizal fungi, reduction in soil alkaline phosphatase activity and available phosphorus content to maize plant. Silver nanoparticles also decrease the mutualistic relation of mycorrhizal fungi and maize plant, which influenced the soil phosphoric cycle and affect soil fertility.

4.5 Effect on Microbial Community Structure and Enzymatic Activities

The microbial community structure is majorly affected by the surrounding environment. Presence of metal nanomaterials in their surrounding directly interacted with microbes and regulate their physiology by positive or negative route. Concentration of nanoparticle is the most important factor, which plays a significant role in growth or inhibition of microbial population and their enzymatic profiles. Different types of enzymatic activity such as soil protease, dehydrogenase, urease, nitrogen fixation,

phosphatase, and ammonia oxidation in soil are directly linked with soil microbial communities. Nanoparticles interaction with these microbial communities interfere with the production of enzymes in soil and impact their optimized production. Therefore, the related enzymatic activity is induced or inhibited (Table 4.2).

He et al. (2011) reported that Fe_2O_3 and Fe_3O_4 magnetic nanoparticles showed positive impact on bacterial population by favoring their growth. They suggested that iron oxide magnetic nanoparticles easily release ions, which bind to natural soil organic compounds like humic and fulvic acids and increase the availability of iron to microbes which promote soil bacterial community.

The enzyme activity of soil microbes is also affected by the presence of nanoparticles but it also depends on interaction with nanoparticles. Joško et al. (2014) observed that the surrounding clay or mineral absorbs the soil microbial extracellular enzymes like urease and phosphatase and protected them from interaction with

Table 4.2 Effect of nanomaterials on soil microbial activities: The nanoparticles interacted with soil microbial communities and influence their growth and physiology, which affect the production of enzymes and their functional role in soil

S. no.	Nanomaterial	Enzyme	Concentration	Effect	Reference
1	Ag	Acid phosphatase, β -glucosidase, Aryl sulfatase, Dehydrogenase and fluorescein diacetate hydrolase	100–1000 mg. kg^{-1}	Reduction in enzyme activity	Shin et al. (2012)
2	Ag	Soil urease and phosphatase activity	At >20 mg. kg^{-1}	Reduction in activity	Rahmatpour et al. (2017)
3	CuO	Urease, phosphates, and dehydroxygenase enzyme activity	100–1000 mg. kg^{-1}	Reduction in activity	Xu et al. (2015)
4	Fe_2O_3	Invertase and urease activity	420–1260 mg. kg^{-1}	Enhancement in activity	He et al. (2011)
5	SiO_2 and Al_2O_3	Ureases, dehydrogenase activity	50 mg. kg^{-1}	No change	McGee et al. (2017)
6	TiO_2	Soil protease, catalase, and peroxidase	4307.5 mg. kg^{-1}	Reduction in activity but increase in urease activity	Du et al. (2011)
7	TiO_2	Urease activity	1 mg. g^{-1}	Decrease	Chai et al. (2015)
8	TiO_2	Nitrogen fixation and methane oxidation	–	Decrease activity	Cai et al. (2014)
9	TiO_2	Soil protease activity	0–2 mg. g^{-1}	Enhancement in activity	Ge et al. (2012)
10	ZVF	Dehydrogenase activity	10 mg. kg^{-1}	Stimulation of dehydrogenase activity	Cullen et al. (2011)

ZVF Zero valent iron nanoparticles

nanoparticles, thus reduced the toxicity on the microbial enzyme activity. In fact, reduction of enzyme activity by metal nanoparticles occurred due to binding of metal to active protein component of enzyme or covering of the active site by the metal, make the unavailability of active reaction site of enzyme to the substrate (Vithanage et al. 2017; Kizilkaya and Bayrakli 2005).

Moll et al. (2016) investigated the effects of multi-walled carbon nanotube (MWCNT), titanium oxide, and cerium oxide on rhizospheric activity in red clove and found that application of nanomaterial did not affect the nitrogen fixation in red clove. They measured increase in nitrogen by 8% in soil treated with MWCNT at 3000 ppm. They also observed the colonization of arbuscular mycorrhiza fungi (AMF) and found no effect of nanoparticles presence on the arbuscular and vesicular colonization. They suggested that the difference in the impact of nanomaterial on plant and microbial activity is species-specific and one type of nanomaterial can affect bidirectional (positive or negative) to different strains. The effect of different types of nanomaterials on enzyme activities and microbial community structure are given below.

4.5.1 Silver Nanoparticles (Ag NP)

Silver nanoparticles are widely used as antibacterial agents in medicine and food packing material. The distribution of silver nanomaterials in soil or wastewater also showed toxicity to nontargeted microbes. It was reported that silver nanoparticles showed inhibition of DNA transcription and enzyme activity, suppression of respiration in planktonic bacteria (Hwang et al. 2008; Choi et al. 2008; Dror-Ehre et al. 2009; Reinsch et al. 2012). Wigginton et al. (2010) and Yang et al. (2014) reported that silver nanoparticles directly interacted with enzymes and influenced the microbial community. Reduction in urease activity was also observed at 100 to 1000 ppm concentration of silver nanoparticles (Shin et al. (2012).

Yang et al. (2013) and Yuan et al. (2013) suggested that silver nanoparticles could impact on the ecosystem productivity and soil fertility by reducing the activity of functional proteins related to nitrogen cycle in *Nitrosomonas europaea*. The detailed information on silver nanoparticles and titanium oxide nanoparticles' impact on microorganism has been reviewed by Schaumann et al. (2015). Previously, Schlich et al. (2013) also studied a modal sewage sludge system contained silver nanoparticles and found that silver nanoparticles showed nano-toxicity toward microorganism. Schlich and Hund-Rinke (2015) also found that silver nanoparticles and silver nitrate are toxic toward ammonia-oxidizing bacteria. The silver nanoparticles reduced the soil respiration and enzymatic activity at 0.14 ppm concentration but at lower concentration 0.0032 to 0.032 ppm did not affect enzymatic activity (Colman et al. 2013; Hänsch and Emmerling 2010). Shin et al. (2012) reported reduction in different enzymes like acid phosphatase, β -glucosidase, aryl sulfatase, dehydrogenase, and fluorescein diacetate hydrolase in sandy soil by citrate-coated silver nanoparticles. Further, Zhai et al. (2016) suggested that continuous increasing

concentration of silver nanoparticles in soil affects the functional composition of microbial communities and related ecosystem. They found that different shapes and concentration of nanomaterial also changed the carbon utilization pattern of present microbial community. Recently, Rahmatpour et al. (2017) also measured significant reduction of soil urease and phosphatase activity at $>20 \text{ mg.kg}^{-1}$ due to Ag nanoparticles. The author revealed that the inhibition of soil enzymatic activity and microbial community structure depends on nanoparticles concentration and soil type. They found no change in soil respiration at low concentration of silver nanoparticles.

4.5.2 Carbon Nanomaterials

The application of carbon nanomaterials has been increased due to their exceptional optical and mechanical properties. The carbon nanomaterials have shown mixed effect on microbial communities. Oyelami and Semple (2015) studied the effect of single- and multi-walled carbon nanotube on soil microbial flora. They found that the amount of glucose in microbial biomass decreased with increasing concentration of carbon nanomaterial in soil but inconsistent with carbon nanomaterial concentration in the range of 1 to 1000 mg.g^{-1} . They observed that increasing concentration does not impact microbial activity in specific pattern. Previously, Tong et al. (2007) also found that at 1– 1000 mg C_{60} fullerenes per kg soil had no impact on total phospholipid content of microbial population after 180 days of incubation. They reported that fullerenes have not changed the microbial community structure and microbial activity at this concentration range.

Further, Nyberg et al. (2008) found no negative impact of C_{60} fullerenes at 50 g.kg^{-1} on microbial community but carbon nanomaterial influenced the food chain through affecting the growth of primary and secondary producer. In contrast, Johansen et al. (2008) reported that C_{60} fullerene reduced the number of fast-growing bacteria which directly influenced the number of bacteria feeding on the protozoan community. They found that fullerene absorbed the essential nutrients like vitamins and minerals from soils, which limited the growth of soil microbes. Chung et al. (2011) also reported that multi-walled carbon nanotubes (MWCNT) reduced the soil enzyme activities at 500 mg.kg^{-1} concentration. Further, Tong et al. (2012) studied the effect of single-walled carbon nanotube (SWCNT) on microbial community structure. They repeatedly used raw SWCNT at $1000 \text{ }\mu\text{g.g}^{-1}$ soil and polyethylene glycol or m-poly amino benzene sulfonic acid functionalized SWCNT at 10 and $50 \text{ }\mu\text{g.g}^{-1}$ soil concentration to challenge *E. coli* and microbial community for 6 weeks. They found that repeated application of SWCNT affected the soil microbial community and soil metabolic activity decreased less with functionalized SWCNT.

Carbon nanomaterial showed both positive and negative impact on microbial community at various concentration levels. It was found that multi-walled carbon nanotubes (MWCNT) at lower concentration in the range of 10– 1000 mg.kg^{-1} showed no remarkable effect on sandy loam soil microbial community but at higher

concentration (1000 mg.kg^{-1}) it reduced *Waddlia*, *Holophaga*, *Derxia*, and *Opitutus* bacterial species but increased *Cellulomonas*, *Rhodococcus*, *Pseudomonas*, and *Nocardioideis* bacterial community (Shrestha et al. 2013).

4.5.3 Copper Oxide Nanoparticles

Copper oxide nanoparticles also affected microbial community with their presence. Gajjar et al. (2009) reported that copper oxide generates free radicals which create toxicity toward bacteria. Similarly, Rousk et al. (2012) also found decay in bacterial growth in mineral soil by copper oxide nanoparticles. Further, Xu et al. (2015) evaluated the impact of copper nanoparticles on soil microbial community at concentration $100\text{--}1000 \text{ mg.kg}^{-1}$ in flooded paddy soil. They measured negative impact on soil microbial biomass, total phospholipid fatty acid, urease, phosphates, and dehydroxygenase enzyme activity. Recently, Simonin et al. (2018) reported that copper oxide nanoparticles negatively affected microbial activity related to nitrogen and carbon cycles in soil. These nanoparticles significantly affected denitrification, nitrification, and soil respiration activities in soil microbes at 100 mg/kg concentration. Further, Joško et al. (2019) found that exposure of copper and zinc nanoparticles in soil for 730 days did not cause significant change in microbial activity at 10 mg.kg^{-1} concentration. They reported that decrease in zinc and copper concentration with time does not mean reduction in their toxicity, but it depends on the type of species in microbial community and their tolerance capacity and adaptation to present engineered nanomaterial in the surrounding environment.

4.5.4 Titanium Oxide Nanoparticles

Titanium dioxide nanoparticles (nano- TiO_2) are the most applicable nanoparticles in personal care industry, food industry as additive, in solar cell as photocatalyst and pigment industry (Tan et al. 2018). The wide use of titanium oxide nanoparticles led their distribution in different environmental sector and their impact on microbial community. It has been reported that TiO_2 nanoparticles retained in soil system for long period and interacted with plants and microbial system (Du et al. 2011). Fang et al. (2010) reported that titanium oxide nanoparticle increased cell permeability by damaging cell membrane of *Nitrosomonas europaea*. In addition to the bacterial community titanium oxide nanoparticles significantly affected the arbuscular mycorrhizal fungal community in soil (Burke et al. 2014).

Titanium oxide nanoparticles showed both positive and negative impact on soil microbial community by reducing and scaling up the microbial diversity (Ge et al. 2011; Shah et al. (2014). Du et al. (2011) found negative impact of titanium oxide nanoparticles in soil enzyme activity. They measured decrease in soil protease,

catalase, and peroxidase but increment in urease activity of soil containing titanium oxide nanoparticles. In contrast, Chai et al. (2015) reported that nano-titanium oxide decreased urease activity. The multiple exposure effect of titanium oxide was also evaluated on microbial nitrifying communities by Simonin et al. (2016). Authors found that titanium oxide nanomaterials affected the diversity of ammonium-oxidizing bacterial communities and negative impact on nitrification enzymes.

Cai et al. (2014) found that titanium oxide declined the nitrogen fixation and methane oxidation by disrupting the gene expression of bacteria but increased the organic material degrading microbial community population. Ge et al. (2012) reported that soil protease activity was found significantly enhanced with the exposure of titanium oxide and zinc oxide nanoparticles due to increase in extracellular protease production by *Streptomyetaceae* and *Streptomyces* class bacteria.

4.5.5 Zinc Oxide Nanoparticles

Zinc oxide nanoparticles have shown their wide application in remediation of pollutant due to photocatalytic activity. Zinc oxide nanoparticles have been applied in different products like paints, plastic, ceramics, glass, cement, rubber, lubricants, pigments, batteries sunscreen creams, and cosmetics. The total volume of zinc oxide nanoparticles is increasing in the environment due to the use of these products (Daughton and Ternes 1999). Zinc is present in the dissolution form (Zn^{2+}) or zinc oxide and in the agglomerated form in the soil or water (Wu et al. 2010). A detailed review on the toxicity of zinc oxide has been covered by Ma et al. (2013). Zinc oxide nanoparticles showed inconsistency in toxicity concentration toward *E. coli* with same size of particles. The zinc oxide nanoparticles in ultrapure water with 20 nm size showed inhibitory concentration (IC_{50}) less than 0.1 ppm to *E. coli*, whereas similar size (10–30 nm) zinc oxide nanoparticles in media showed IC_{50} 500 mg.l^{-1} to wild *E. coli* (Li et al. 2011; Premanathan et al. 2011). 100% inhibition of *E. coli* has been reported by 10–70 nm particle size zinc oxide nanoparticle (Brayner et al. 2006; Jiang et al. 2009 and Liu et al. 2009).

The coating on nanoparticles also affected the toxicity of nanoparticles. It has been reported that zinc oxide showed less toxicity in the presence of tannic acid in comparison to alginic, fulvic, and humic acid (Li et al. 2010). They reported that the presence of organic acid reduced the bioavailability of free Zn^{2+} ions in surround matrix. Further, Li et al. (2011) proved that the presence of free Zn^{2+} is mainly responsible for their toxicity by experimentation of different media types. Ge et al. (2011) found that zinc oxide nanoparticles decrease the microbial biomass and soil bacterial diversity and significantly change the soil enzymes activity. In addition to bacteria, zinc oxide nanoparticles also showed toxicity to yeast. In the short-term experiment, Kasemets et al. (2009) found that within 24 h exposure, 30–70 nm sized zinc oxide nanoparticles showed more toxicity in comparison to bulk zinc oxide to the yeast *Saccharomyces cerevisiae* with EC_{50} of 131 and 158 ppm. While, Lipovsky et al. (2011) found only 1 ppm zinc oxide nanoparticles inhibited 95% growth of

pathogenic yeast, *Candida albicans*. It was hypothesized that the toxicity of nanoparticle depends on solubilization of zinc from zinc oxide nanoparticles in the medium.

Zinc oxide nanoparticles also showed toxicity to various algal species such as *Skeletonema marioni*, *Thalassiosira pseudonana*, *Dunaliella tertiolecta*, and *Isochrysis galbana* at 1 ppm concentration. Miller et al. (2010) measured 50–75% decrease in growth rate of these algae by 20–30 nm sized ZnO nanoparticles at 1 ppm concentration. Similarly, it showed toxicity to microalgae *Pseudokirchneriella subcapitata*, diatom *Thalassiosira pseudonana*, and cyanobacteria *Anabaena flos-aquae* (Franklin et al. 2007; Miao et al. 2010; Brayner et al. 2010).

4.5.6 Iron Nanoparticles

Iron is also an important micronutrient for plant growth promotion and microbial metabolism. The high concentration of iron in the soil directly impacted the physiology of plant and microbial community and disorganized the soil ecosystem by transforming microbial community structure. He et al. (2011) found that Fe₂O₃ nanoparticles induced the invertase and urease activity at 420–1260 ppm but bacterial biomass did not change. Similarly, Cullen et al. (2011) reported that at 10 ppm concentration zero valent iron nanoparticles affected microbial enzymatic activity by stimulation of dehydrogenase activity, inhibition of microbial ammonia oxidation, and no impact on hydrolase activity.

4.5.7 Silicon and Aluminum Oxide Nanoparticles

The effect of silicon dioxide and aluminum oxide nanoparticles on microbial community is very rare. Recently, McGee et al. (2017) reported that silicon oxide and aluminum oxide nanoparticles did not alter the soil ureases, dehydrogenase activity and soil microbial community but silver nanoparticles reduced that enzyme activity significantly. On the exposure of silver nanoparticles, abundance of Proteobacteria increased but Acidobacteria and Verrucomicrobia decreased.

4.5.8 Nano-Ceria (CeO₂)

Microbial activity related to carbon, nitrogen, and phosphorus cycle is affected by the presence of nano-ceria (Hamidat et al. 2016). Authors used different sized (3.5 or 31 nm) citrate-coated ceria nanoparticles to evaluate their activity in rhizospheric soil at 1 mg.kg⁻¹ concentration. They found decrease in microbial enzymatic activity and alteration in bacterial community structure. Citrate coating on nano-ceria

reduced the negative impact on microbial enzymatic activity in comparison to pristine ceria nanoparticles (Hamidat et al. 2016).

4.6 Effect on Water Microbial Flora

The use of nanoparticle-based consumer products and their release in water bodies is also increasing their concentration in water bodies. The nanoparticles also affect the microbial community structure and microbial physiology in water bodies. The effect of nanoparticles on freshwater bacteria in three Swedish lakes was studied by Farkas et al. (2015). They found reduction at 1000 ppm concentration in the abundance of freshwater bacteria. The activity of nanoparticles in water also depends on the pH, dissolved titanium oxide organic matter, and ionic strength, and also influences particle stability (Christian et al. 2008, Keller et al. 2010, Ottofuelling et al. 2011). Qiu et al. (2016) also revealed toxicity of titanium oxide nanoparticles on bacterial community in wastewater system but overall biodiversity was remained unchanged. They found that production of extra polymer substance prevents the toxic effect of titanium oxide nanoparticles by removal and deposition as sludge from the system. Further, Hou et al. (2012) and (2013) reported that zinc oxide and silver nanoparticles in wastewater inhibited the nitrifying bacteria *Rhodocrobium*, *Pseudomonas*, and δ -Proteobacteria species by reducing their respiration. Das et al. (2012) also reported the effect of carboxy functionalized silver (Ag) nanoparticles on natural water microbial community and found significant change in bacterial community and reduced bacterial biomass. The citrate and Gum Arabic-coated Ag nanoparticles reduced the chemical oxygen demand and ammonia removal efficiency in wastewater microbial communities at 0.2–2 ppm. The decrease of microbial diversity was also assessed by Alito and Gunsch (2014). Further, Puay et al. (2015) showed that toxicity of copper oxide nanoparticles in microbial communities of wastewater. In contrast, on exposure of nanomaterial some microbes produce extracellular polymers which reduce the toxicity by chelating metal nanoparticles.

4.7 Mechanism of Nanomaterial Toxicity to Microbial Community

The toxicity of nanoparticles to microorganism is found via different mode of actions such as formation of reactive oxygen species (ROS), interruption of energy transduction, disruption of ionic channels, and inhibition of enzyme activity (Xia et al. 2008). Mainly toxicity depends on the dissolution of nanomaterial. The dissolution of nanomaterial also affected with the different environmental factors such as pH, temperature, presence of organic matter, as well as physiochemical properties of material such as size, shape, surface area, and chemical composition. It has

been found that small size nanoparticles have higher dissolution rate in comparison to bigger particles (Meulenkamp 1998; Bian et al. 2011; Mudunkotuwa et al. 2011; Reed et al. 2012).

It has been reported that nanoparticles internalized into bacteria or it absorb on the surface or associate with the membrane (Brayner et al. 2006; Kumar et al. 2011; Dimkpa et al. 2012). The toxicity of nanoparticles toward bacteria was assessed in reduction of colony forming unit, optical density measurement, and calculation of minimum inhibitory concentration in many reports at monoculture system. But, at community level, omics approach was used to provide genomic, proteomic, and metabolomics information to understand the toxicity of nanoparticles at ground level. Kaweeteerawat et al. (2015) reported toxicity of different metal oxide nanoparticles to *Escherichia coli*. They observed the growth inhibition by CoO, Co₃O₄, Cr₂O₃, CuO, Mn₂O₃, Ni₂O₃, and ZnO metal oxides due to membrane damage and oxidative stress in the cell.

4.7.1 Reactive Oxygen Species (ROS) Production

Production of reactive oxygen species is the major cause of microbial damage. The ROS react on phospholipids in cell membrane and oxidize the double bond in the plasma membrane which increase membrane flexibility and permeability (Cabiscol et al. 2010). Further, ROS damage the DNA by cross-linking and strand break in bacteria. ROS can also disturb the proteins by development of sulfide bond between sulfur-containing amino acids (Imlay 2003). In many reports, it has been reported that UV radiation induces the production of ROS in bacteria (Kumar et al. 2011, Lu et al. 2012). The effect of C₆₀ fullerene and carbon nanotubes (CNTs) on bioluminescent marine bacterium *Vibrio fischeri* was studied by Chae et al. (2011). They found that under UV-A irradiation, ROS production and microbial inactivation increased. The increase in ROS production under UV-A illumination decreased the respiration rate, which is measured by degradation of 2-chlorophenol. In contrast, Lyon and Alvarez (2008) found no toxicity of C₆₀ fullerene by photocatalytic ROS production. They reported that the toxicity of C₆₀ fullerene is due to oxidation of membrane lipids and proteins without ROS production.

4.8 Conclusion

The increasing utilization of nanomaterial-based consumer products and attraction of human beings toward nanomaterial-based technology demand the higher production of nanomaterials. Increasing production also increases the release rate of such nanomaterials into the environment, where they interact with the microbial community. The interaction of microbes and metal nanomaterial affects the microbial community structure and physiology. The reduction or enhancement of microbial

diversity depends on concentration of metal nanoparticles and their chemical composition. The reduction of microbial community also depends on associated microbial type. The metal tolerant capacity and production of extra cellular polymers reduce the nano-toxicity. Silver, zinc oxide, titanium oxide, and carbon-based nanoparticles have been widely studied nanomaterials, which showed mixed effect on diverse microbial community in soil and water ecosystem. The generation of reactive oxygen species, DNA damage, penetration of nanoparticles into the microbial cell, and disorganization of cellular membrane are the major causes of microbial reduction. Most of studies on nano-toxicity showed mixed effect on microbial community at short-term exposure but understanding of nano-toxicity effect on microbial community at long-term exposure required more research.

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Chapter 5

Nanomaterials Causing Cellular Toxicity and Genotoxicity



Bensu Karahalil

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Abstract Nanomaterials have been commonly used in many applications in society, and their use is increasing year by year. Therefore, it is essential to make the risk assessment of these materials. Most important toxicologic effects such as genotoxic and mutagenic effects need to be meticulously evaluated because these effects are related to major health concerns such as cancer and inherited genetic damage. There are different methods used to test genotoxic effects of nanomaterials namely comet assay, micronucleus assay, chromosome aberration test, bacterial and mammalian mutagenicity tests. There are some interactions between nanoparticles and some solutions or chemicals or reagents used in these assays. Furthermore, the nanomaterials of different substances and their toxicity are evaluated by using toxicology assays. The in vivo genotoxicity studies found in the literature for some of the most common nanoparticles of metallic and nonmetallic substances were compiled in this review, that is, aluminum oxide, copper oxide, iron oxide, zinc oxide, titanium oxide, and carbon-based nanomaterials, silica. There are conflicting results for these materials and their reasons explained. It is also emphasized the importance of administration route of nanomaterials in genotoxicity studies, and target tissue statement especially is important for inhalation toxicity. We need the benefits of

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V. Kumar et al. (eds.), *Nanotoxicology and Nanoecotoxicology Vol. 1*, Environmental Chemistry for a Sustainable World 59, https://doi.org/10.1007/978-3-030-63241-0_5

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nanotechnology; however, nanoparticles are managed by using the risk assessment methodology.

Keywords Nanoparticles · Genotoxicity · Risk assessment

5.1 Introduction

Nanotechnology is a very fast-growing field. There are many nanotechnology applications ranging from medical diagnostics and prognostics to issues of environmental sciences because of their unique properties (Nath and Banerjee 2013). Manufactured nanomaterials are used in many commercially available consumer products, such as cosmetics, packaging, paints, sunscreens and semiconductors and textiles. They are also used in medical applications to increase the quality of life enabling early diagnosis and treatment of diseases. Due to the increasing production volumes, thereby more exposure to nanomaterials, environmental exposure to these materials is inevitable (Contado 2015).

Nanomaterials have unique features such as small size, composition, surface structure, solubility, shape, and aggregation. These features allow for limitless modifications of their basic properties such as controlled drug release, solubility, diffusion, targeting, stability, half-life in circulatory system. Nanomaterials have advantages over their large-scale counterparts due to having unique properties, and they are preferred in many areas (Jennifer and Maciej 2013).

Due to both the increasing production of nanomaterials and growing dissemination of information on nanomaterials, synthetic nanomaterials should be evaluated for their potential environmental impact/hazard potential prior to their use in products because of the inevitable release of their waste materials into the environment (Ray et al. 2009). There are currently relatively scanty data on the toxicity of nanomaterials to environmentally relevant species, limiting the quantitative risk assessment of nanomaterials. Due to the increased production of synthetic nanomaterials, the occupational and public exposure to nanomaterials is expected to increase dramatically in the coming years as well as their potential release in the environment (OECD International Futures Programme). As mentioned above, nanomaterials are used not only in medicine, drug and food industries, but also they have potential environmental applications such as remediation of contaminated groundwater with iron nanoparticles.

Although already significant amount of toxicological information concerning nanoparticles is available, ecotoxicological data on nanoparticles are relatively newly emerging. This toxicological information is obtained at various biological levels, such as from *in vitro* cell culture studies to *in vivo* animal studies. In contrast more data and experience have been relatively more readily available on bulk chemicals in the evaluation of environmental hazard compared to individual particles since it requires a more specific and exhaustive analysis to evaluate individual

particles (Kahru and Dubourguier 2010). Three key elements of nanomaterials toxicity screening strategies have been outlined by Oberdörster et al. (2005a, b):

- (1) Physicochemical characterization (size, surface area, shape, solubility, and aggregation)
- (2) Elucidation of biological effects involving in vitro (cellular and noncellular)
- (3) In vivo studies (Oberdörster et al. 2005b)

Physicochemical characteristics of nanoparticles (solubility, surface area, chemical composition, etc.) have major impacts to show the toxicity. For example, poorly soluble nanoparticles cause cancer and exhibit more toxicity. Greater potential hazard may relate to the surface area of nanoparticles compared with larger sized particles. Chemical composition and absorption rate on surface of nanoparticles have important impact to show their toxicity. Size of nanoparticles is a key factor for determining their toxicity. If the particle is very small, its surface area-to-volume ratio is much greater and also chemical reactivity and biological activity are also much higher. Aggregation of nanoparticles with cells, surface charges, and morphology of nanoparticles are important parameters for health effects (Viswanath and Kim 2016).

The effects of nanoparticles have been studied on organisms such as algae and fish. Two endpoints are used, namely, the biomarkers of effect and the biomarkers of exposure, including growth and survival rate, mobility and reproduction. Dose, distribution, bioavailability, fate and degradation of nanoparticles are used to assess the exposure of nanoparticles. Risk assessment is very important for (1) creating awareness of hazards and risk; (2) identifying organisms who may be at risk such as fish; (3) determining existing control preventions or if needed further augment; (4) determining whether a control program is required for a particular hazard; (5) providing data for legal regulations. Risk assessment is made by integration of hazard identification, risk analysis, and risk evaluation. For risk assessment, available literature is searched from databases and has information on toxicity of nanoparticles. A dose-response relationship is established by exposure information. Assessment of exposure is analyzed by assays (in vitro tests; tissue and cell cultures, animal toxicity tests, and epidemiological studies) which are biomarker of exposure and biomarker of effect. These assays are applied to in vitro, in vivo, or cell cultures. Similar studies are compared with each other; risk characterization is performed according to the results from exposure to econanoparticles and biomarker evaluation. Thus, if needed, protective preventions are taken and exposure are minimized or removed (CCOHS 2018; Kuempel et al. 2012). Due to the difficulty of conducting epidemiological studies, toxicological biomarkers in molecular epidemiology studies are used more extensively in the risk characterization. For hazard identification of nanoparticles and to demonstrate the toxicity of nanoparticles, cellular toxicity and genotoxicity tests should be carried out. It is important to keep in mind that toxicity assessments of nanoparticles because of their unique properties differ from larger-sized particles since these differences may cause problems in the conducting of toxicity tests. Some technical problems may occur such as agglomeration and interactions between nanoparticles and biological cell components in different media.

There are many studies conducted with econanoparticles to show their toxicity. A pioneering study of Oberdörster (2004) showed that C60 fullerenes were inducing changes in the brain of the fish already at very low aquatic exposure level. Namely, significant lipid peroxidation was found in brains of largemouth bass after 48 h of exposure to 0.5 mg/l of uncoated C60 fullerenes (tetrahydrofuran was used for solubilization of C60) (Oberdörster 2004).

Nanoparticles have been studied for cell toxicity, immunotoxicity, and genotoxicity. Human are exposed to various nanoscale materials since childhood, and the new emerging field of nanotechnology has become another threat to human life (Oberdörster et al. 2005a, b). Because of their small size, nanoparticles find their way easily to enter the human body and cross the various biological barriers and may reach the most sensitive organs. (Pourmand and Abdollahi 2012). Nanoparticles can easily enter into the cells and membranes. They do accumulate in tissues; therefore, they interact easily with cells via chemical reaction. They can be transferred easily from one cell to another cell. As the result of this journey, they can induce the increase of free radical and reactive oxygen species (ROS) caused by oxidative stress due to their unique physicochemical characterization.

Although numerous studies have demonstrated different toxic effects associated with nanomaterials such as oxidative stress, mitochondrial damage, chromosomal aberrations and oxidative DNA damage, altered cell cycle regulation, and protein denaturation, we still have little information on their toxicity mechanisms. One of the most frequently suggested mechanisms about their toxicity is the production of reactive oxygen species (ROS) and development of oxidative stress which are responsible for damaging biomolecules such as DNA, RNA, proteins, and lipids (Valdiglesias et al. 2015).

5.1.1 Toxicity of Nanoparticles

There has been an increasing public concern regarding the potential toxicity implications of nanoparticles. It is important to develop tests to evaluate the safety and tolerability of nanoparticles after exposure of biological systems SCENIHR 2006. When the possible toxicity of NPs take place after, commonly, inhalation or oral intake, predominantly lung and gastroenteral toxicities studies have been investigated. Inhalation is a common route for exposure to nanomaterials. Thus, much research has been performed on the pulmonary toxicity tests caused by nanoparticles.

5.1.2 Nanoparticles of Metallic Substances

The toxic effects of test substances are usually measured in terms of acute, sub-acute, subchronic or chronic exposure conditions. A maximum of 2 weeks (14 days), a maximum of 4 weeks (28 days), a maximum of 13 weeks (90 days) and longer

than 4 months are normally referred to as acute, subacute, subchronic, and chronic toxicity studies, respectively (Chen et al. 2008; Radziun et al. 2011; Alshatwi et al. 2012; Balasubramanyam et al. 2009; Kim et al. 2009).

Appropriate toxicological examinations of risks and benefits of nanomaterials are still rare. Toxicological interactions of water soluble metals or their compounds depend on the chemical properties. Physicochemical characteristics of metals and their interactions with cells affect biological outcomes and action mechanisms. Copper (Cu) is an essential trace element. Cellular Cu homeostasis is regulated under physiological conditions however Cu is toxic under overload conditions due to generating the highly reactive hydroxyl radical (OH $\cdot$) by Fenton reaction which cause damage to biomolecules such as DNA, RNA, proteins, and lipids. Cu-based nanoparticles have been shown cytotoxic and genotoxic effects compared to copper based micro-sized particles. Copper oxide (CuO) nanoparticle induced higher extent of DNA damage than CuO microparticles (MP) in the comet assay. CuO nanoparticle increase chromosomal damage as determined by micronuclei formation (Boyles et al. 2016).

5.2 Iron Oxide Nanoparticles (FeO)

Iron, cobalt and nickel nanoparticles are known as magnetic nanoparticles because of their stability and magnetic features. There are several types of iron oxide (FeO) nanoparticles, such as hematite (α -Fe $_2$ O $_3$), magnetite (γ -Fe $_2$ O $_3$), and magnetite (Fe $_3$ O $_4$). FeO nanoparticles are commonly used and there are widespread application fields, especially human imaging and early recognition of disease, with the use of specific nanoagents for molecular imaging in the context of Magnetic Resonance Imaging (MRI), ultrasound, optical imaging, and X-ray imaging (Indira and Lakshmi 2010). Magnetic nanoparticle is biocompatible and biodegradable, also can be used in drug field, such as drug delivery, gene delivery, and targeting. There are some health concerns on FeO nanoparticles because of their widespread application. Many toxicological studies have been carried out on FeO nanoparticles; however, it is still unclear whether they are generally safe or should be used carefully. One of the toxic effects of FeO nanoparticles is oxidative damage caused by Reactive Oxygen Species production due to their high surface area to volume. Oxidative damage to the cell membrane is due to releasing of intracellular enzymes such as lactate dehydrogenase, whereas reduced glutathione (GSH) is one of the most important barriers against oxidative damage. FeO nanoparticles induced cytotoxicity in mammalian cells. However, little is known about the genotoxicity of IONPs following exposure to human cells. The cytotoxicity, oxidative stress, and genotoxicity of FeO nanoparticles in two human cell lines (skin epithelial A431 and lung epithelial A549) are investigated. It was shown that FeO nanoparticles induced dose-dependent cytotoxicity and oxidative stress in both types of cells, which was demonstrated by cell viability and lactate dehydrogenase leakage assays. FeO nanoparticles also cause the depletion of glutathione and induction of reactive

oxygen species and lipid peroxidation. FeO nanoparticles increase the DNA damage which was shown by Comet assay, and it was shown that the expression levels of mRNA of caspase-3 and caspase-9 genes were higher (Ahamed et al. 2013).

5.3 Zinc Oxide Nanoparticles (ZnO Nanoparticles)

ZnO nanoparticles possess ultraviolet scattering, antibacterial, and antifungal properties. Because of bactericidal properties, they are commonly used on biomedical applications and food packaging, and also cosmetics, textiles, and electronic products. Due to the increased Zn exposure, toxicity and safety of Zn nanoparticle are challenges to use ZnO nanoparticles (Ng et al. 2017). Cytotoxicity mechanisms of ZnO nanoparticles are not well understood. The generation of free radicals and production of reactive oxygen species from the surface of ZnO are major components for cytotoxicity. When cells exposure to ZnO nanoparticles, defense systems in cells are activated to remove or minimize the toxic effects of nanoparticles (Namvar et al. 2015). Oxidative damage and antioxidant balance are important, if the balance is destroyed, oxidative harm occurs (Poljsak et al. 2013) and can cause cell death. Teng et al. examined the cytotoxicity of ZnO nanoparticles at various concentrations in MRC5 cells and found a significant morphological change in ZnO nanoparticle-treated MRC5 cells compared to control cells. They also observed the association between ZnO nanoparticle-treated MRC5 cells and the release of LDH in a dose-dependent manner, especially 50 µg/mL ZnO nanoparticle exposure could cause cell death. They also showed genotoxicity of ZnO nanoparticles. ZnO nanoparticle s exposure causes reactive oxygen species production, leading to an accumulation of 8-hydroxy-2'-deoxyguanosine (8-OHdG). Oxidative DNA damage due to 8-OHdG accumulation was detected by the comet assay (Ng et al. 2017). Exposure to ZnO nanoparticles in the acidic environment such as in the lung lining fluid causes ZnO dissolution. This type of exposure provides the transient increases in the concentration of Zn²⁺ ions and causes local toxicity. Inhalation exposure and instillation ZnO nanoparticles of rats in a dose-dependent manner resulted in transient inflammation measured in the bronchoalveolar tissues. There are few studies which have investigated neurotoxicity of ZnO. Vandebriel and De Jong administered ZnO nanoparticles (20–80 nm) intraperitoneally to rats (4 mg/kg bw) for 8 weeks (ip 20–80 nm/twice weekly) and showed that spatial learning and memory ability were attenuated by alteration of synaptic plasticity in rats (Vandebriel and De Jong 2012).

5.4 Titanium Dioxide Nanoparticles (TiO₂ Nanoparticles)

Titanium dioxide (TiO₂) nanoparticles are commonly used for drug delivery systems, antibacterial materials, cosmetics, sunscreens, and electronics. Nanoparticles generally possess dramatically different physicochemical properties compared to fine particles (FPs). Traditionally, TiO₂ FPs have been considered as poorly soluble, low toxicity particles, so they are used as a “negative control” in toxicological studies. Rats are exposed to high concentration of TiO₂ FPs for 2 years and lung tumors developed therefore, the International Agency for Research on Cancer (IARC) classified TiO₂ as a Group 2B carcinogen (possibly carcinogenic to humans). However, the tumorigenic effect of TiO₂ FPs has been questioned and attributed to lung overload rather than specific carcinogenicity of fine TiO₂ (Shi et al. 2013). Sager et al. investigated toxicity of both TiO₂ nanoparticles (80/20 anatase/rutile; 21 nm, P-25) and TiO₂ FPs (100% rutile; 1µm) in rats. They showed that nano TiO₂ was 40-fold more potent in inducing lung inflammation and damage at 1 and 42 days after exposure than fine TiO₂ on an equal mass burden. However, respective potencies were not significantly different when dose was expressed on the basis of total surface area of particles delivered to the lung (Sager et al. 2008).

Genotoxicity of titanium dioxide (TiO₂) nanoparticles have been investigated in numerous studies. Comet assay and micronucleus test have been applied to show DNA damage of TiO₂ nanoparticles in different cell cultures and animal models. The standard comet assay, not enzyme-modified comet assay, is able to discriminate between the genotoxicity of different types of TiO₂. According to the standard comet assay, anatase TiO₂ is one of the strongest genotoxic type among TiO₂ nanoparticles. It correlates with their photocatalytic activities (Møller et al. 2017). Many studies (in vivo and in vitro) were carried out to investigate the genotoxicity of TiO₂ FPs and nanoparticles, but results are conflicting. Some studies showed that TiO₂ nanoparticles are genotoxic, whereas the others not. The reason for different results may be due to the use of different nanoparticle sizes, particle dispersion, exposure metrics, crystalline structure, and cell types may be an explanation (Shi et al. 2013).

Anatase TiO₂-NPs cause greater responses, a reduction of cell viability, an increase of inflammatory indices (e.g., lactate dehydrogenase, LDH, interleukin-8, IL-8) and an increase in Radical Oxygen Species (ROS) generation. Therefore, it induces the cell death by an intrinsic apoptosis pathway (Iavicoli et al. 2011). Studies have revealed that TiO₂ nanoparticles are more toxic than FPs in different animal models and multiple exposure routes such as inhalation, dermal exposure, intratracheal instillation, oral gavage, intragastric, intraperitoneal, or intravenous injection Oberdorster 2001; Oberdorster et al. 1994; Shakeel et al. 2016; Liu and Yang 2013; Grissa et al. 2015). There is no information on acute toxicity for TiO₂ nanoparticles in humans. A value often given in animal toxicity studies is the median lethal dose (LD50)/median lethal concentration (LC50), which is defined as the dosage/concentration resulting in the death of 50% of the experimental animals (Shi et al. 2013).

Chang et al. carried out meta-analysis to investigate toxicity of nano-TiO₂ and its potential harmful impact on human health. 62 of 375 articles were selected after applying the inclusion criteria for the meta analysis study. Data were retrieved in vitro and from short-time animal studies and according to included and excluded criteria from 1994 to 2011. Different parameters for toxicity were used in cell, animal, and rat models, such as apoptotic and necrotic modifications, DNA damage, genotoxicity, increases in cytokines IL-6 and IL-8, cytotoxicity and inflammation, reactive oxygen species, apoptosis, lung toxicities and presence of aggregates or agglomerates, liver DNA cleavage, and hepatocyte apoptosis. The combined toxic effects of TiO₂ nanoparticles were calculated by the different endpoints by cell and animal models. They observed that more than 50% showed positive statistical significance except the apoptosis group. The cytotoxicity was in a dose-dependent but was not clear in size-dependent manner (Chang et al. 2013).

5.4.1 Nanoparticles of Nonmetallic Substances

Carbon-Based Nanomaterials

Carbon-based nanomaterials are used in different sectors since they have limited reactivity and wide surface area. They are stable and strong antioxidants. Among them fullerenes and carbon nanotubes are widely used ones. It is shown that cytotoxicity of single-wall carbon nanotubes is higher than multiwall carbon nanotubes (MWCNTs) in a study performed using a guinea pig alveolar macrophage. Single-wall carbon nanotubes considerably impaired phagocytosis of macrophages at low dose (0.38 µg/mL), while multiwall carbon nanotubes and C60 induced injury only at a high dose (Jia et al. 2005). Solubility of fullerene derivatives are one of the key parameters to show cytotoxicity. Single-wall carbon nanotubes were tested for toxicity on human embryo kidney cells (HEK293) and it was demonstrated that they inhibit the proliferation of these cells by inducing cell apoptosis and decreasing cellular adhesive ability (Chawla and Kumar 2013). When cells were exposed to single-wall carbon nanotubes, toxic effects were found both dose-dependent and time dependent manner and single-wall carbon nanotubes induce oxidative stress in biological systems. In aquatic environment, single-wall carbon nanotubes have toxic effects in fish such as gill irritation and brain injury. Studies indicated that carbon nanotubes induce pulmonary toxicity and inflammation showed in mice (Junyi 2015).

Reproductive toxicity of carbon nanotubes has been also investigated. Carbon nanotubes studies of single-wall carbon nanotubes and double-wall carbon nanotubes biological effect on embryonic development of model species of *Danio rerio* (zebrafish) reveal significant embryonic development inhibition but this effect occurred with 2 times higher concentrations of double-wall carbon nanotubes (Cheng et al. 2007). Fluorescently labeled multiwall carbon nanotubes injected to *Danio rerio* embryos and they spread to the whole blastoderm. They did not show any anomalies however the next generation of *Danio rerio* from multiwall carbon

nanotubes exposed ones had less viability indices. A recent review suggested that the placenta does not provide a tight barrier against the transfer of nanoparticles to fetuses, specifically against the distribution of carbonaceous nanoparticles to and in the fetus. (Ema et al. 2016).

Silica Nanoparticles

Comprehensive production and use of silica nanoparticles have increased the risk of human exposure. For this reason, safety of silica nanoparticles is a major issue. Silica nanoparticles are widely used in construction, catalysis, paints (pigments) and pharmaceutical applications, agriculture, food, and consumer products. The health effects of silica, especially crystalline silica (0.5–10 μ m) on human have widely been studied. Occupational exposure to crystalline silica induces a fibrotic lung disease, also called silicosis, in workers and also causes emphysema and pulmonary tuberculosis. On the contrary, natural amorphous silica is generally less harmful than crystalline silica (Murugadoss et al. 2017).

Animal inhalation studies were carried out with silica nanoparticles to assess the effects of silica nanoparticles on blood biochemical parameters and changes on pathology and histology. Silica nanoparticles caused the transient changes on breathing parameters, increased lung weight, total bronchoalveolar lavage cells and proteins, induced acute inflammation and tissue damage. Liver is key tissue for silica nanoparticles toxicity. The lactate dehydrogenase (LDH) level in exposed mice was found significantly higher than controls, and the alanine transaminase (ALT) levels were also increased. All these elevation as indicate cell membrane injury and tissue damage. For aspartate aminotransferase (AST), it was observed little elevation and no any increase was observed with serum albumin, blood urea nitrogen (BUN), and creatinine levels. Lymphocytic infiltration, granuloma formation, and hydropic degeneration in the hepatocytes were observed in the livers. It indicated that the silica nanoparticles may be hepatotoxic. Pulmonary hyperemia and pulmonary interstitial thickening were observed (Yu et al. 2013).

Silica nanoparticles caused hepatotoxicity however possible mechanisms of hepatotoxicity still remain unclear. Ahmad et al. investigated the toxic effects of silica nanoparticles on liver. They designed a series of experiments on human liver cell line HepG2 to clarify the hepatotoxicity mechanism of silica nanoparticles. They explored that silica nanoparticles (14 nm) causes apoptosis via reactive oxygen species. Silica nanoparticles also leads to oxidative stress through reactive oxygen species production and lipid peroxidation and thereby depletion of glutathione. Whereas mRNA and protein expressions of cell cycle checkpoint gene p53 and apoptotic genes (bax and caspase-3) were upregulated and antiapoptotic gene was down regulated in exposed mice. All these results contributed to hepatotoxicity mechanisms of silica nanoparticles (Ahmad et al. 2012).

Nanoparticles of Polymeric Materials

Polymer-based nanoparticle is a collective term which is given to any kind of polymer-based nanoparticle, but specifically is applied for nanospheres and nanocapsules. Polymer-based nanoparticle is used and have roles in photonics, electronics, sensors, medicine, pollution control, and environmental technology (Mallakpour

and Behranvand 2016). Especially, it is widely used in medicine due to their specific physical and chemical properties which alter the normal biological activity, as compared to bulk materials. The main use of polymer-based nanoparticle is in drug delivery, although they are also used in bioimaging and biosensing assays. Cytotoxicity and degradation by-products are major problems and it needs to be further investigated in order to improve the biocompatibility of polymeric nanoparticles. Regarding this, many polymeric core-shell nanoparticles are being explored in various clinical phase trials, meaning that they have so far surpassed the cellular and animal toxicity requirements (Moreno-Vega et al. 2012, Table 5.1).

Table 5.1 Nanomaterials of different substances and their toxicity

Nanomaterials of different substances	Toxicity	References
<i>Nanoparticles of metallic substances</i>		
Aluminum oxide	Disturb the cell viability, alter mitochondrial function, increase oxidative stress, alter tight junction protein expression of blood brain barrier, genotoxic (comet and micronucleus test assay) Positive for cell viability by MTT ^a assay, negative for cell viability by EZ4U assay ^b	Chen et al. (2008), Radziun et al. (2011), Alshatwi et al. (2012), Balasubramanyam et al., (2009) and Kim et al. (2009)
Copper oxide	Cytotoxicity in numerous cell types apoptosis occurring concomitantly with genotoxicity and oxidative stress DNA damage (comet assay, CAs, micronucleus test)	Bryce et al. (2007), Berntsen et al. (2010), Studer et al. (2010), Huang et al. (2006) and Seiffert et al. (2011), 25:140–152
Iron oxide	Cytotoxicity Fe2O3 (MTT) Cell viability reduced (LDH leakage assay, HepG2 cells) Exposure time increases, cell viability reduces. DNA damage (phosphorylated H2AX) Oxidative damage	Sadeghi et al. (2015) Indira and Lakshmi (2010)
Zinc oxide	Morphological changes in MRC cells, 8-OHdG accumulation, inflammation, neurological dysfunction (reduced spatial learning and memory ability)	Ng et al. (2017) and Vandebriel and De Jong, (2012)
Titanium oxide	Lung inflammation and damage, genotoxic by standard comet assay, cell death, apoptosis, cytotoxicity in a dose-dependent manner	Sager et al. (2008), Moller et al. (2017), Shi et al. (2013) and Chang et al. (2013)

(continued)

Table 5.1 (continued)

Nanomaterials of different substances	Toxicity	References
<i>Nanoparticles of nonmetallic substances</i>		
Carbon-based nanomaterials	Impaired phagocytosis of macrophages, reproductive toxicity, oxidative toxicity, pulmonary toxicity	Jia et al. (2005), Chawla and Kumar. (2013) and Junyi (2015)
Silica	Fibrotic lung disease (silicosis), hepatotoxicity, pulmonary toxicity	Murugadoss et al. (2017), Yu et al. (2013) and Ahmad et al. (2012)
<i>Nanoparticles of polymeric materials</i>	Cytotoxicity	Moreno-Vega et al. (2012)
Poly butyl cyanoacrylate nanoparticles	MTT viability test in HeLa and HEK cells False positive results have been described caused by molar extinction of the NPs	Dhawan and Sharma (2010)
Tween80 nanoparticles	Decrease cell viability	Voigt et al. (2014)

MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

^aThe MTT assay is a colorimetric assay for assessing cell metabolic activity

^bEZ4U assay, Cell proliferation and cytotoxicity assay. All Nanoparticles in the table causes the reduced cell viability and reactive oxygen species production

5.5 Conclusions

In this report, the most common assays for testing genotoxicity of NMs have been described. Most important nanoparticles of metallic and nonmetallic substances are listed, and their toxic effects are presented by toxicity assays used. The benefit of physicochemical properties of nanoparticles and also their effects on health is discussed. Physicochemical characteristics of nanoparticles (solubility, surface area, chemical composition, etc.) have major impacts to show the toxicity. Inhalation is a common route for exposure to nanomaterials. Therefore, it is essential to perform target-specific assays and also carefully evaluate the toxicity on lung and related respiratory organs.

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Chapter 6

Exploring Microbial Nanotoxicity Against Drug Resistance in Bacteria



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Abstract The growing threat of antimicrobial resistance on human health urgently calls for the need to look for novel solutions to mitigate the grave effect of this global problem and save thousands of lives each year. Nanotechnology is an emerging area that is expected to be able to have solutions toward containing the rise and spread of multidrug-resistant microorganisms. The present chapter focuses on understanding the potential of nanomaterials to cope with drug-resistant bacteria. While different types of nanoparticles have emerged as nanoweapons against drug-resistant or multidrug-resistant bacteria, other nanomaterials like quantum dots, nanotubes, dendrimers, fullerenes, and nanoparticle-conjugated antimicrobial peptides or antibiotics are being explored in parallel for their antimicrobial properties. The chapter looks at interactions between nanomaterials and drug-resistant microbes

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both at the physical and molecular levels to analyze their future potential as significant alternatives to antibiotics.

Keywords Nanoparticles · Nanotoxicity · Drug-resistant bacteria · Antimicrobial resistance · Multi-drug resistance

6.1 Introduction

The unprecedented rise of drug-resistant bacteria is posing a grave threat to public health. Resistance against several classes antibiotics has also paved way for increased prevalence of multidrug resistance in bacteria, leading to adverse health outcomes (Ebinesh 2017). Microbial drug resistance or antimicrobial resistance (AMR), driven by misuse of antimicrobials in humans and animals, has resulted in them becoming more and more ineffective. This is further manifested by longer patient-recovery periods, extended hospital stays, and costlier treatment processes (Perron et al. 2015). Moreover, increasing rates of AMR could also potentially undo many of the medical advances made globally (Tanwar et al. 2014).

The World Health Organization has highlighted AMR as one of the top ten threats to global health. The first report of the Global Antimicrobial Resistance Surveillance System outlines alarmingly high levels of resistance in common infection-causing bacteria (World Health Organization 2017). In 2016, 490,000 people are said to have developed multidrug-resistant tuberculosis globally (Tacconelli et al. 2018). Drug resistance is also beginning to complicate HIV and malaria treatments (Goldberg et al. 2012). It is reported that at least 23,000 people in the United States died of infections caused by antibiotic-resistant bacteria (Centers for Disease Control and Prevention 2017). If not addressed urgently, AMR is estimated to lead to almost ten million deaths and economic losses worth 100 trillion dollars by the year 2050 (O'Neill 2016).

The scenario above calls for newer strategies and solutions to tackle the burden of drug-resistant infections. These include approaches such as discovery of new antimicrobials or alternatives to kill bacteria, modification of existing antibiotics, development of antimicrobial peptides, or introducing vaccination as a preventive measure against infections. The use of the antimicrobial properties of nanoparticles has also shown a promising potential (Hemeg 2017; Rudramurthy et al. 2016; Sinha and Khare 2014). The toxic effect of nanoparticles on bacteria has been well documented. Metal nanoparticles, metal oxide nanoparticles, and quantum dots have been shown to inhibit growth of common infection-causing bacteria such as *E. coli*, *P. aeruginosa*, *S. aureus*. (Adams et al. 2014; Guzman et al. 2012; Lara et al. 2010b; Sinha et al. 2011). Variation in nanotoxicity levels has been observed with the nanoparticle shape, size, and concentration (Lu et al. 2013; Tam et al. 2008; Yamanaka et al. 2005).

Historically, silver nanoparticles have been commonly explored as effective biocidal agents against a wide range of bacteria (Marambio-Jones and Hoek 2010; Rai

et al. 2012). Cytotoxic effects of metal oxide nanoparticles like zinc oxide, titanium dioxide, copper oxide, aluminum oxide, silica, and magnesium oxide have been observed (Besinis et al. 2014; Butler et al. 2014; Frenk et al. 2013; Ge et al. 2012; Sinha and Khare 2014; Wang et al. 2010). The bactericidal activity of nanoparticle is possibly caused by reasons such as disruption of cell membrane architecture/permeability, nanoparticle internalization and accumulation in the cytoplasm, interaction of nanoparticle with cellular proteins and DNA, etc. Antimicrobial properties of organic nanoparticles such as quaternary ammonium compounds, quaternary ammonium polyethylenimines, chitosan nanoparticles, and polycationic nanoparticles have also been reported (Beyth et al. 2015).

Developments in nanotechnology have therefore facilitated the design and application of nanoparticles as a new line of defense against drug-resistant microorganisms (Khameneh et al. 2016; Singh et al. 2014). In view of this, the chapter focuses on understanding the recent developments demonstrating the potential of nanomaterials to cope with drug resistance in bacteria. It also throws light on different approaches like use of functionalized nanoparticles, antibiotic–nanoparticle conjugates, quantum dots, and antimicrobial peptide–nanoparticle conjugates for effective action against multidrug-resistant bacteria and for newer evolutions in nanomedicine. An understanding of the resistant microbe–nanoparticle interactions as well as their mechanism of action will aid design and development of novel nanomaterial-based strategies of antimicrobial resistance containment.

6.2 Effect of Nanoparticles on Drug-Resistant Bacteria

The most commonly investigated nanoparticles for their effectiveness against resistant bacteria are metal and metal oxide nanoparticles. Nanoparticles show a wide variation in size, ranging between 5 and 96 nm, and in shapes (spherical, triangular, polygonal, or even aggregates). Some of the common drug-resistant bacteria against which nanotoxic effects have been shown include *S. aureus*, *P. aeruginosa*, *E. coli*, *M. luteus*, *A. baumannii*, *Klebsiella* sp., *Bacillus* spp., and *E. faecalis*. Antimicrobial properties of organic nanoparticles have been studied, but there have been few reports on their toxic effects against drug-resistant or multidrug-resistant bacteria. This section discusses the antimicrobial effects of chemically synthesized, biologically synthesized, and functionalized nanoparticles on bacteria resistant to drugs or antibiotics.

6.2.1 Effects of Chemically Synthesized Nanoparticles on Drug-Resistant Bacteria

The bactericidal effects of chemically synthesized or commercially manufactured metal or metal oxide nanoparticles on drug-resistant bacteria are shown in Table 6.1. These include inhibition of bacterial growth, reduced number of viable bacteria, concentration-dependent antibacterial effects, and reduction in biofilm formation efficacy. Majority of the studies appear to have been documented for silver nanoparticles, while other nanoparticles such as magnesium oxide, zinc oxide, and iron oxide have also been investigated for their antibacterial potential on resistant bacteria. Wan et al. (2016) reported the effectiveness of silver nanoparticles in causing complete inhibition of carbapenem-resistant *A. baumannii*. The study also establishes that a combination of silver nanoparticles with antibiotics could be an effective means of inhibiting carbapenem-resistant *A. baumannii*, at reduced and less toxic doses. Biofilms are constituted by an aggregate of microorganisms in which cells adhere to each other or to a surface with a self-produced matrix of extracellular

Table 6.1 Effect of chemically synthesized nanoparticles on drug-resistant bacteria

Nanoparticle	Nanoparticle shape and size	Resistant bacteria	Observation	Reference(s)
Silver	10, 30–40, 100 nm	Methicillin-resistant <i>S. aureus</i>	Inhibition of bacterial growth in a bactericidal manner	Ayala-Nunez et al. (2009)
			10-nm nanoparticle most effective over other sizes at same dose	
Silver	100 nm	Multidrug-resistant <i>P. aeruginosa</i> , ampicillin-resistant <i>E. coli</i> O157:H7, erythromycin-resistant <i>S. pyogenes</i>	Bactericidal effect	Lara et al. (2010b)
			Inhibition of bacterial growth rate since first contact with nanoparticles	
Magnesium oxide	<30 nm	<i>S. pneumoniae</i>	Maximum inhibition at 10- μ g nanoparticle concentration	Gokulakrishnan et al. (2012)
			Inhibition of bacterial growth from second hour onwards at 10- μ g concentration	

(continued)

Table 6.1 (continued)

Nanoparticle	Nanoparticle shape and size	Resistant bacteria	Observation	Reference(s)
Superparamagnetic iron oxide nanoparticles (SPION)	9.92 ± 3.14 nm	Methicillin-resistant <i>S. aureus</i> (Mu50 strain)	Decreased planktonic growth and viable bacteria counts	Durmus et al. (2013)
			81% increase in antibiofilm efficacy in the presence of fructose metabolite	
			Eradicated methicillin-resistant <i>S. aureus</i> biofilms better than vancomycin	
Silver	Spherical, 5–12 nm	Carbapenem-resistant <i>A. baumannii</i>	Growth of <i>A. baumannii</i> completely inhibited at 2.5 µg/mL	Wan et al. (2016)
			Nanoparticle showed synergistic effects with polymyxin B and rifampicin; additive effect with tigecycline	
Silver	Spherical, 11 nm	Multidrug-resistant <i>S. aureus</i> and <i>P. aeruginosa</i>	Time- and dose-dependent antibacterial effects of nanoparticles	Yuan et al. (2017)
Magnesium oxide	Spherical, size <100 nm	<i>E. coli</i> , <i>K. pneumoniae</i> and <i>S. aureus</i>	Accelerated rate of membrane disruption in the presence of nanoparticles	Hayat et al. (2018)
			Time-dependent reduction in biofilm formation potential	
			Significant reduction in biomass of 48–120 hr old biofilms	

(continued)

Table 6.1 (continued)

Nanoparticle	Nanoparticle shape and size	Resistant bacteria	Observation	Reference(s)
Zinc oxide	Spherical, 30 nm	Carbapenem-resistant <i>Acinetobacter baumannii</i>	Good antibacterial activity	Tiwari et al. (2018)
Iron oxide	Round, 10 nm	Ampicillin- and kanamycin-resistant <i>E. coli</i>	Potential antibacterial activity	Gabrielyan et al. (2019)

polymeric substances. The efficacy of nanoparticles against antibiotic-resistant biofilms has also been shown. Superparamagnetic iron oxide nanoparticles in combination with fructose metabolites were explored as a new antimicrobial strategy, which showed an 81% increase in antibiofilm efficacy (Durmus et al. 2013). Similarly, in vitro antibiofilm and antiadhesion effects of magnesium oxide nanoparticles were also observed by Hayat et al. (2018).

6.2.2 Effect of Biologically Synthesized Nanoparticles on Drug-Resistant Bacteria

The biological synthesis of nanoparticles is an innovative approach that provides for a simpler, cost-effective, nonpathogenic, and more environment-friendly process of nanoparticle synthesis over chemical synthesis processes. Bacterial, fungal, and plant-based resources have been used for the bio-fabrication of nanoparticles and their effects on drug-resistant bacteria seen (Table 6.2). The effects of nanoparticles include dose-dependent inhibition of bacterial growth, antibiofilm and antibacterial activity, cell damage and death, and loss of cell viability. As observed earlier, silver nanoparticles are among those widely studied. Antibacterial activity of antibiotics was enhanced when impregnated with biologically synthesized silver nanoparticles (Naqvi et al. 2013). The nanoparticle-impregnated antibiotics were able to exert antibacterial effects on eight multidrug-resistant bacteria isolates. Similarly, two-drug combination of bio-silver nanoparticles and oregano essential oil was found to be much more effective against multidrug-resistant strains, over individual treatments (Scandorieiro et al. 2016). Antibiofilm formation by resistant bacteria was also inhibited by biologically synthesized silver nanoparticles (Ali et al. 2018; Das et al. 2017a; Kanmani and Lim 2013).

Table 6.2 Effect of biologically synthesized nanoparticles on drug-resistant bacteria

Nanoparticle	Size and shape of nanoparticle	Biological synthesis process	Resistant bacteria	Observations	Reference(s)
Silver	Spherical, 5–30 nm	Extracellular mycosynthesis using culture filtrate of <i>Aspergillus flavus</i>	Eight multidrug-resistant isolates: <i>E. coli</i> , <i>P. aeruginosa</i> , <i>E. faecalis</i> , <i>S. aureus</i> , <i>M. luteus</i> , <i>A. baumannii</i> , <i>K. pneumoniae</i> , and <i>Bacillus</i> spp.	Antibacterial activity of imipenem, gentamicin, vancomycin, and ciprofloxacin augmented upon being impregnated with silver nanoparticles	Naqvi et al. (2013)
				Synergistic effect of antibiotics and nanoparticles led to a 0.2–7.0-fold increase in antibacterial activity	
Silver	Spherical, 55–83 nm	Synthesized using leaf extract of <i>Mimusops elengi</i> , Linn.	Multidrug-resistant <i>K. pneumoniae</i> , <i>M. luteus</i> , and <i>S. aureus</i>	Maximum antibacterial activity against <i>K. pneumoniae</i> and moderate activity against <i>M. luteus</i> , <i>S. aureus</i>	Prakash et al. (2013)
Silver	Spherical, 2–15 nm	Synthesized using exopolysaccharide extracted from <i>L. rhamnosus</i> GG	Multidrug-resistant <i>P. aeruginosa</i> and <i>K. pneumoniae</i>	Dose-dependent inhibition of bacteria	Kammani and Lim (2013)
				Both bacteria were more susceptible to nanoparticles than <i>L. monocytogenes</i>	
				Dose-dependent biofilm inhibition	
				100% inhibition of biofilm formation by <i>P. aeruginosa</i> at 56 µg/ml nanoparticle concentration	

(continued)

Table 6.2 (continued)

Nanoparticle	Size and shape of nanoparticle	Biological synthesis process	Resistant bacteria	Observations	Reference(s)
Silver	Spherical, 24 ± 8 nm	Synthesized using <i>P. amarus</i> extract	Multidrug-resistant <i>P. aeruginosa</i>	Excellent antibacterial potential exhibited	Singh et al. (2014)
Copper and zinc oxide	Copper nanoparticle: Spherical, 12–90 nm, Zinc oxide nanoparticle: Aggregate form, 16–96 nm	Synthesized from nonpathogenic <i>E. faecalis</i> by extracellular enzymatic method	Methicillin-resistant <i>S. aureus</i> 20	Efficient antibacterial and antibiofilm activity of both nanoparticles	Ashajothi et al. (2016)
Silver	77.68 nm	Synthesized using <i>F. oxysporum</i> , strain 551	Methicillin-resistant <i>S. aureus</i> strains N315, 101, and 107, carbenemase- producing <i>E. coli</i> (<i>E. coli</i> KPC 131 and 133), carbenem-resistant <i>A. baumannii</i>	Growth of drug-resistant and multidrug-resistant strains inhibited In combination with oregano essential oil, bio-silver nanoparticles showed much lower minimum inhibitory concentration values compared to individual treatment Combination treatment caused a faster reduction in colony-forming units per milliliter than individual treatments	Scandorio et al. (2016)
Zinc oxide	Spherical, 22.5 nm	Synthesized using leaf extract of <i>Aristolochia indica</i>	Multidrug-resistant <i>P. aeruginosa</i> , <i>A. baumannii</i> , <i>E. coli</i> , and methicillin-resistant <i>S. aureus</i>	Strong bactericidal properties Both strains significantly inhibited	Stieff et al. (2017)

Silver	Triangular, 18 ± 3 nm (SEM); 16 ± 2 nm (TEM)	Synthesized from leaf extract of <i>Ocimum gratissimum</i>	Multidrug-resistant <i>E. coli</i> (MC-2)	Inhibition of biofilm formation	Das et al. (2017a)
				Damage to cell surface and production of reactive oxygen species	
				Dose-dependent antimicrobial activity	
				Antibiofilm properties of HP-silver nanoparticles	
Silver	Spherical, 13.15 nm	Synthesized using bark extract of the medicinal plant <i>Holarthra pubescens</i> Wall ex G. Don (HP)	Imipenem-resistant <i>P. aeruginosa</i>	Nanoparticle-induced cell damage and cell death	Ali et al. (2018)
				Silver nanoparticles inhibit or restrict colonization and halt biofilm formation	
				Potential antibacterial property	
Silver	Spherical, 41–68 nm	Synthesized from the <i>Bacillus brevis</i> (NCIM 2533)	Multidrug-resistant <i>S. typhi</i> and <i>S. aureus</i>		Saravanan et al. (2018)
Zinc oxide phytonanocomposite	10–20 nm	Synthesized using <i>Strychnos nux-vomica</i> L. (<i>Loganiaceae</i>) leaf extract	Methicillin-resistant <i>S. aureus</i> , multidrug-resistant <i>E. coli</i> , <i>P. aeruginosa</i> , <i>A. baumannii</i> (isolated from diabetic foot ulcers)	Significant antimicrobial activity	Steffy et al. (2018)

(continued)

Table 6.2 (continued)

Nanoparticle	Size and shape of nanoparticle	Biological synthesis process	Resistant bacteria	Observations	Reference(s)
Zinc oxide	17–30 nm	Synthesized using <i>Camellia japonica</i> plant leaf extract	Extended-spectrum β lactamase-positive <i>E. coli</i> and <i>P. mirabilis</i>	Nanoparticles have stronger antibacterial effect against strains	Maruthupandy et al. (2018)
				Loss of cell viability, shrinkage of cell, membrane disarrangement, cell wall lysis observed in strains	
Selenium	Spherical, 55 nm	Synthesized using <i>Aspergillus oryzae</i> -fermented Lupin extract and gamma radiation	Wide range of multidrug-resistant bacteria and fungi	Broad-spectrum antimicrobial activity	Mosallam et al. (2018)

6.2.3 Effect of Functionalized Nanoparticles on Drug-Resistant Bacteria

Functionalization of nanoparticles using different types of ligands enables multivalent interactions and stronger adherence to biological molecules. As a result, such functionalized nanoparticles have also demonstrated nanotoxic effects against resistant bacteria (Table 6.3). Surface functionalization of nanoparticles has been carried out by different approaches such as linking to side chains or capping with polymeric agents. Capping of silver nanoparticles with carboxymethyl tamarind prevented self-aggregation and enabled long-term nanoparticle stabilization (Sanyasi et al. 2016). These nanoparticles effectively inhibited the growth of multidrug-resistant bacterial strains *S. haemolyticus*, *S. epidermis*, *E. coli*, *K. pneumoniae*, and *E. cloacae*, in comparison to commonly used antibiotics. Very recently, silver nanoparticles have been developed having hydroxyapatite–zoledronate functional groups. These were found to be active against multidrug-resistant Gram-positive and Gram-negative bacteria (Boanini et al. 2018). Alcohol-functionalized silver nanoparticles were active against antibiotic-resistant *E. coli*, *S. aureus*, and *S. typhimurium* (Dorjnamjin et al. 2008). Similarly, silver nanoparticles modified with polyvinyl alcohol–melamine formaldehyde were able to inhibit infections caused by antibiotic-resistant strains (Kakkar et al. 2015).

Table 6.3 also shows that in addition to silver, zinc oxide nanoparticles have also been functionalized quite frequently and used for their antibacterial effect against a range of resistant bacteria. Zinc oxide alginate beads exerted 98% antibacterial effect over resistant *E. coli* DH5- α (Baek et al. 2019a). In another interesting study, gold nanoparticles modified with cationic functional groups were found to remarkably inhibit the bacterial resistance for as many as 20 generations (Li et al. 2014). Gold nanoparticles have also been functionalized with enzymes or amino acids (Tiwari et al. 2011, Chen et al. 2010). Functionalized metal oxide nanoparticles such as alumina-coated iron oxide magnetic nanoparticles and Fe₃O₄–gold nanoeegs have also been reported for their antimicrobial efficacy against multidrug-resistant bacteria (Malka et al. 2013; Huang et al. 2009; Yu et al. 2011).

It is also interesting to note that metal-doped nanoparticles have also shown promising antimicrobial features against resistant bacteria (Kunkalekar et al. 2013, Malka et al. 2013, Hameed et al. 2016). Antimicrobial activity of the zinc-doped copper oxide nanocomposite on methicillin-resistant *S. aureus* and multidrug-resistant *E. coli* was 10,000 times more as compared to individual nanoparticles (Malka et al. 2013). A comparative study on extended-spectrum beta-lactamase-producing strains of *E. coli* and *K. pneumoniae* showed that neodymium doped zinc oxide nanoparticles had greater antibacterial effect than pure zinc oxide nanoparticles (Hameed et al. 2016). Das et al. (2017b) have also reported the disinfection of multidrug-resistant *E. coli* using iron-doped zinc oxide nanoparticles. When used as a photocatalyst for disinfection, the nanoparticles induced lipid peroxidation and potassium ion leakage indicating membrane disintegration.

Table 6.3 Effect of functionalized nanoparticles on drug-resistant bacteria

Nanoparticle	Nanoparticle shape and size	Resistant bacteria	Observation	Reference
Gold–lysozyme nanoclusters	2.3 ± 0.3 nm	Pan-drug-resistant <i>A. baumannii</i> and vancomycin-resistant <i>E. faecalis</i>	Cell growth effectively inhibited	Chen et al. (2010)
Cationic and hydrophobic functionalized gold (NP3, with most hydrophobic end group)	2 nm	Multidrug-resistant <i>E. coli</i> , <i>E. cloacae</i> complex, <i>P. aeruginosa</i> , <i>S. aureus</i> , and methicillin-resistant <i>S. aureus</i>	Effective suppression of growth of 11 clinical multiple drug-resistant isolates	Li et al. (2014)
			Low toxicity to mammalian cells	
			Bacterial resistance was not observed after 20 generations	
Gum arabic-capped silver	Spherical, 5–10 nm	Multidrug-resistant biofilm-forming <i>P. aeruginosa</i>	Antibiofilm efficacy	Ansari et al. (2014b)
			Severely deformed and damaged cells	
			Concentration-dependent inhibition of bacterial growth	
Polyethyleneimine-capped zinc oxide	–	Range of multiple antibiotic-resistant Gram-negative bacterial strains, tetracycline-resistant <i>E. coli</i> MREC33	Better antibacterial activity than uncapped nanoparticle	Chakraborti et al. (2014)
			Capped nanoparticle at LD ₅₀ and in combination with tetracycline inhibited growth of tetracycline-resistant <i>E. coli</i> MREC33 by 80%	
Aloe vera extract-capped zinc oxide	Spherical, oval, and hexagonal, 8–18 nm	Methicillin-resistant <i>S. aureus</i>	Significant antibacterial activity observed	Ali et al. (2016)
			Nanoparticles inhibited bacterial growth, exopolysaccharide, and biofilm formation	

(continued)

Table 6.3 (continued)

Nanoparticle	Nanoparticle shape and size	Resistant bacteria	Observation	Reference
Carboxymethyl tamarind polysaccharide-capped silver	Spherical and polygonal, 20–40 nm	<i>S. haemolyticus</i> , <i>S. epidermidis</i> , <i>E. coli</i> C19, <i>K. pneumoniae</i> Kp52, <i>E. cloacae</i> Ec18	Effective inhibition of bacterial growth at concentrations of ~1.5 µg/ml	Sanyasi et al. (2016)
			Nontoxic against mammalian cells	
Polyvinylpyrrolidone-capped silver	133 nm	Carbapenem-resistant strain of <i>A. baumannii</i>	Antibacterial activity at 30-µM concentration; no cytotoxic effect on human pulmonary cell line	Tiwari et al. (2017)
			80% decrease in viability of intracellular bacteria	
			Decreases adherence of <i>A. baumannii</i> to A-549 cell line and intracellular concentration of <i>A. baumannii</i>	
Jacalin-copper sulfide (JCuS) complex	–	Gram-positive (<i>S. aureus</i> , <i>B. subtilis</i>) Gram-negative (<i>E. coli</i> , <i>A. hydrophila</i>) strains	Enhanced antibacterial activity	Ahmed et al. (2018)
Zinc oxide–alginate beads	Irregular spherical shape; 120–236 nm	<i>Escherichia coli</i> DH5-α, <i>Pseudomonas aeruginosa</i>	Antibacterial effect with 98% and 88% removal rate in case of <i>E. coli</i> DH5-α, <i>P. aeruginosa</i> respectively	Baek et al. (2019a)
Zinc oxide and titanium dioxide-conjugated carbon nanotube and graphene oxide nanohybrids	–	<i>E. coli</i> DH5α	Zinc oxide-conjugated nanohybrids exhibited a higher antibacterial property than others	Baek et al. (2019b)
			Significant damages to cell membranes	

6.3 Mechanism of Nanoparticle-Mediated Toxicity to Control Antibiotic-Resistant Bacteria

The mechanism of nanoparticle-mediated control of antibiotic resistance in bacteria is a relatively new area of research and various studies are being carried out to understand bacterial response to nanoparticles. The various types of approaches which are possibly responsible for nanoparticle-assisted damage to the bacterial cells have been discussed in detail below.

(i) Effect on Bacterial Membrane

The bacterial membrane forms the first barrier to nanoparticles. Previously conducted studies have found that Gram-positive bacteria are more prone to damage by nanoparticles as compared to Gram-negative ones. This difference is due to the cell wall structure in both types of bacteria. The Gram-negative bacteria have an additional outer layer containing lipopolysaccharides which confers resistance to most of the drugs and other hydrophobic compounds (Hajipour et al. 2012). Gram-positive bacteria, on the other hand, possess a thick peptidoglycan-layered cell wall (≈ 80 nm thick) with covalently attached teichoic acids. This protective layer is comparatively less complex both physically and chemically as compared to the Gram-negative bacteria where an outer membrane is present.

Various studies have shown the effects of different types of nanoparticles on the membrane. For instance, Liu et al. (2009) found that zinc oxide nanoparticles caused deformation of the membrane and led to the leakage of the intracellular components of *E. coli*. In another study, the effect of titanium dioxide nanoparticles was studied on *E. coli* membrane and a similar damaged membrane resulting in the leakage of cellular components was observed. Based on these results, the possible mechanism of nanoparticle-mediated membrane damage was elucidated by means of a fluorescent dye, 1-N-phenylnaphthylamine. The uptake of this dye was increased with an increase in the concentrations of nanoparticle exposure indicating membrane rupture (Ranjan and Ramalingam 2016).

Since silver nanoparticles have been extensively used as antibacterial agents, their mechanism of action has been proposed by recently conducted studies. These nanoparticles dissolve in the presence of H_2O_2/O_2 which results in their easy uptake by the cellular membrane under the influence of the proton motive force (AshaRani et al. 2008; Choi and Hu 2008). It has also been studied that exposing the cells to millimolar concentrations of silver nanoparticles leads to severe morphological changes including shrinkage of cytoplasm and subsequent detachment of cell membrane leading to further degradation. This results in the leakage of intracellular components. The above-described changes curb the growth of the bacteria. Studies conducted in the recent past show the interaction of *E. coli* cells with silver nanoparticles. As revealed by transmission electron microscope images, the silver nanoparticles first adhere to the membrane and then penetrate within. Further, silver nanoparticles with oxidized surfaces lead to the formation of large holes in the membrane of *E. coli* and a substantial part of the cellular membrane seems to be lost

(Ramalingam et al. 2016). Silver nanoparticles have also reportedly led to the formation of irregular-shaped pits in the outer membrane of *E. coli*, resulting in leakage of lipopolysaccharide molecules and proteins (Kaweeteerawat et al. 2017). The detrimental effects of silver nanoparticles have also been reported for *P. aeruginosa*, *V. cholera*, and *S. typhus* (Dos Santos et al. 2014; Franci et al. 2015). Not only silver nanoparticles but also silver nanocomposites as well as silver nanoparticles modified with various surfactants interact with the bacterial membrane (Dallas et al. 2011).

(ii) Effect on Enzymes/Other Relevant Proteins

The interaction of nanoparticles on various proteins/enzymes is an important area to understand the behavior of their action. It is documented that silver nanoparticles react with sulfur-containing proteins present on the cell surface (Rudramurthy et al. 2016). Silver ions which are formed by the ionization of silver and its compounds have affinity for various proteins and amino acids. Similarly, gold nanoparticles also get attached with proteins as functional moieties (Baptista et al. 2008). This helps in the increase in their antimicrobial efficacy.

Many drug-resistant bacteria remain functional in the presence of antibiotics due to the presence of efflux pumps (Gupta et al. 2017). These efflux pumps are membrane transporter proteins and hence they help the bacteria to survive by extruding the drug out of the cell. Nanoparticles have been possibly known to inhibit these efflux pumps thereby inhibiting drug-resistant bacteria. A study was conducted to investigate the bactericidal effect of zinc oxide nanoparticles on drug-resistant *S. aureus* (Banoee et al. 2010). These nanoparticles were specifically targeted against the NorA efflux pump of *S. aureus*. A considerable increase in the zone of inhibition was observed for ciprofloxacin in the presence of zinc oxide nanoparticles indicating their ability to inhibit the efflux pump. Meanwhile, studies have also been conducted to coat or modify the surface of the nanoparticles to improve their efficacy toward antibiotic-resistant bacteria. For example, polyacrylic acid-coated FeO nanoparticles when administered along with rifampicin to *Mycobacterium smegmatis* showed a four-fold increase in the growth inhibition as compared to the antibiotic alone, suggesting the efflux inhibitory role of the modified nanoparticles (Padwal et al. 2014). To further probe into the mechanism of nanoparticle-mediated inhibition of efflux pumps, it was proposed that these probably bind to the active site as competitive inhibitors of efflux pumps, thereby inactivating them, and hence their ability to extrude the antibiotic out of the cell is impeded (Padwal et al. 2014). Another mechanism which has been put forward is that these nanoparticles function as efflux pump inhibitors by interfering with the efflux kinetics. Earlier studies also proposed the disruption of the proton gradient by nanoparticles, which leads to the disturbance of the proton motive force, thereby leading to a loss in the activity of efflux pump (Choi et al. 2008; Dibrov et al. 2002).

Besides the antibacterial activity, nanoparticles are also being used for antiviral activity as reported in studies (Huy et al. 2017; Lara et al. 2010a). For example, silver nanoparticles in combination with the chitosan composites displayed antiviral activity against H1N1 influenza virus (Mori et al. 2013). The study proposed that one of the major factors responsible for the inhibitory activity of silver

nanoparticles could be the modification of viral proteins by destroying their disulfide linkages. On similar lines, the copper iodide nanoparticles were also found to possess high antiviral activity against feline calicivirus (Shionoiri et al. 2012). This was possibly due to reactive oxygen species-mediated oxidation of capsid proteins. Hence, nanoparticles have been proven to work in a systematic manner to curb the growth of drug-resistant microbes and viruses by targeting various types of proteins.

(iii) Antioxidative Potential

The production of reactive oxygen species under the influence of nanoparticles becomes yet another major mechanism for their inhibitory effect on bacteria. One of the commonest modes of action of silver nanoparticles is their inhibition of respiratory enzymes leading to the generation of reactive oxygen species (Khalandi et al. 2017). Zinc oxide nanoparticles have been known to generate reactive oxygen species such as OH^- and H_2O_2 (Soren et al. 2018). H_2O_2 in particular gets penetrated via the bacterial membrane and leads to growth inhibition. The other types of reactive oxygen species including superoxides and OH^- do not have the ability to enter the bacterial membrane and hence their mode of action differs from H_2O_2 . Besides silver and zinc oxide nanoparticles, Fe_3O_4 has also been studied with the aim to find its mechanism of inhibition and the possible generation of reactive oxygen species like superoxide radicals (O_2^-), singlet oxygen ($^1\text{O}_2$), hydroxyl radicals ($\text{OH}^\cdot$), and H_2O_2 (Auffan et al. 2008). Very recently, titanium nanoparticles have been proven to induce the formation of reactive oxygen species in *E. coli*. This study was conducted by using 2,7-dichlorofluorescein diacetate which fluoresces under oxidative conditions. The reactive oxygen species estimated to be produced by disturbance in the electron transport chain was primarily responsible for depolarization of the cell membrane leading to damage of the cell wall (Ranjan and Ramalingam 2016).

Some studies even suggest that the oxidation state of the metal nanoparticles is also a major factor in determining their bactericidal effect (Meghana et al. 2015). For example, copper dioxide nanoparticles are more toxic than copper oxide nanoparticles since they lead to increased levels of reactive oxygen species like OH^- and H_2O_2 . However, it is not very clear whether the damage caused by nanoparticle-mediated reactive oxygen species formation is the primary or the secondary mechanism of disturbance/toxicity (Meghana et al. 2015). An increasing number of studies have also shown that reactive oxygen species exert oxidative stress by interaction with DNA and proteins in bacterial cells. They have been found to attack the essential periplasmic proteins/enzymes which are required for maintaining the various physiological cell processes (Jahnke et al. 2016). Nanoparticle-induced reactive oxygen species have also been shown to enhance oxidative stress by regulating the expression of proteins paving the way for cell apoptosis.

The exact mechanism of nanoparticle-induced reactive oxygen species production has been a topic of great interest among many other studies. The first and the well-studied mechanism is through photocatalytic means where light induces the formation of highly reactive reactants in the nanoparticles (He et al. 2013). For example, H_2O_2 or OH^- , after interaction with zinc oxide nanoparticles, gets oxidized to $\text{OH}^\cdot$ (hydroxyl radical). This free radical after interaction with O_2 and zinc

oxide gets converted to the most damaging superoxide radical (O_2^-) (Zhang et al. 2010). On the other hand, the reactive oxygen species generation due to ultrasonication has also been recently reported. Ultrasonic activated zinc oxide nanoparticles have the ability to split H_2O into H^+ which further interacts with O_2 to generate H_2O_2 (Ansari et al. 2016). Thus, reactive oxygen species generation under the effect of nanoparticles is an upcoming research area to study their cytotoxic activity against drug-resistant microorganisms. Table 6.4 summarizes the mechanism of action of different nanoparticles on drug-resistant bacteria.

6.4 Advances in Addressing Antimicrobial Resistance by Nanoparticle-Mediated Approaches

The potential of nanoparticles in controlling the spread of multidrug-resistant bacteria has opened tremendous scope for their exploitation. Among the different types of nanoparticles, silver nanoparticles have shown immense success in biomedical applications (Chaloupka et al. 2010). Their mechanism for reducing the growth of clinically relevant resistant strains has now been studied in detail. Other nanoparticles like those of titanium dioxide, iron oxide, and copper oxide have also been studied for their antibacterial properties; however, certain drawbacks like colloidal instability and aggregation problems have limited their usage in biological applications (Baalousha et al. 2008; Degabriel et al. 2018). Nevertheless, recent new research is now focusing to modify nanoparticles by attaching functional groups or other compounds like antibiotics to enhance their efficacy toward multidrug-resistant bacteria. Some of these latest approaches are being discussed in the current section.

(i) Modification of Carbon Nanotubes

Carbon nanotubes and their functionalized variants have been deemed to hold great promise in the fight against multidrug-resistant bacterial infections (Maleki Dizaj et al. 2015; Mocan et al. 2016). Surface modifications in carbon nanotubes have yielded many desirable effects in the recent past. A study was conducted where in multi-walled carbon nanotubes were synthesized modified with silver nanoparticles. These nanostructures were found to be quite effective against pathogens like *E. coli* and *S. aureus* due to disruption in their membrane function leading to cell death (Dinh et al. 2015). Similarly PEGylated silver-coated single-walled carbon nanotubes were found to be effective in controlling the growth of food pathogen *Salmonella* sp. (Chaudhari et al. 2015).

A recent study showed the antibacterial activity of functionalized single-walled carbon nanotubes synthesized using herbal extracts of *Hempedu bumi*. These carbon nanotubes were potent against pathogenic bacteria like *E. coli* and *Bacillus* sp. (Foo et al. 2018). Wang et al. (2017) showed that the modification of multi-walled carbon nanotubes with OH, COOH, and NH_2 groups led to an increased antifungal

Table 6.4 Mechanism of action of different nanoparticles on drug-resistant strains

Nanoparticle	Targeted bacteria	Mechanism of action	Reference
Aluminum oxide	<i>E. coli</i>	Cell wall damage, enters cytoplasm	Ansari et al. (2014a)
Silver nanoparticles impregnated with titanium dioxide films	<i>E. coli</i> , <i>S. pyogenes</i> , <i>S. aureus</i> , <i>A. baumannii</i>	Reactive oxygen species production	Wong et al. (2015)
Iron oxide	<i>P. aeruginosa</i> and <i>S. aureus</i>	Disruption of the bacterial membrane	Ismail et al. (2015)
Nickel	<i>E. coli</i> , <i>K. pneumoniae</i> , <i>S. typhi</i> , <i>B. subtilis</i> , <i>S. epidermidis</i>	Reactive oxygen species production, release of free ions, membrane damage, inhibition of electron transport	Jeyaraj Pandian et al. (2016)
Titanium dioxide	<i>Salmonella typhimurium</i> , <i>E. coli</i>	Membrane rupture by reactive oxygen species generation	Ranjan and Ramalingam (2016)
Graphene oxide nanosheets	<i>E. coli</i>	Cause stress on cell membrane leading to an increase in reactive oxygen species finally causing bacterial death	Zhang et al. (2016)
Silver	<i>Acinetobacter baumannii</i> , <i>Salmonella typhi</i> , <i>Vibrio cholerae</i> , <i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , multidrug-resistant <i>Escherichia coli</i> , <i>Streptococcus pyogenes</i> , <i>Pseudomonas aeruginosa</i> , coagulase-negative <i>Staphylococcus epidermis</i> , <i>Enterococcus faecalis</i> , <i>Klebsiella pneumoniae</i> , <i>Listeria monocytogenes</i> , <i>Proteus mirabilis</i> , <i>Micrococcus luteus</i>	Reactive oxygen species generation, lipid peroxidation, membrane damage, cell wall synthesis inhibition, increase in membrane permeability, damage to lipids and proteins, ribosome destabilization, intercalation between DNA bases, biofilm disruption	Dakal et al. (2016), El Din et al. (2016), and Franci et al. (2015)
Zinc oxide	<i>S. aureus</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>B. subtilis</i> , <i>Stenotrophomonas acidaminiphila</i> , methicillin-resistant <i>Streptococcus agalactiae</i> , <i>Enterobacter aerogenes</i> , <i>E. coli</i> , <i>Klebsiella oxytoca</i> , <i>S. aureus</i> , <i>S. pyogenes</i>	Reactive oxygen species generation, biofilm, enhanced membrane permeability, disintegration of membrane, adsorption to cell surface, damage of lipids and proteins	Esparza-González et al. (2016) and Sirelkhatim et al. (2015)
Ampicillin-coated gold	<i>Staphylococcus aureus</i>	Reactive oxygen species-mediated damage	Silvero et al. (2018)

activity like inhibition of spore formation, germination against pathogenic strain *Fusarium graminearum* (Wang et al. 2017). In an interesting observation, nanoshell-mediated laser therapy using multi-walled carbon nanotubes exerted “localized killing effects” on bacteria (Mocan et al. 2016). Here, multi-walled carbon nanotubes were covalently functionalized with immunoglobulin G. Using immunoglobulin G, multi-walled carbon nanotubes followed by laser irradiation on methicillin-resistant *Staphylococcus aureus*, led to increased death rates.

(ii) Modifications of Fullerenes

Fullerenes are 60 carbon-containing nanostructures having great potential in the biomedical sector (Bartelmess and Giordani 2014). Functionalization of fullerenes has been attempted in the recent past with the aim to increase their cytotoxic potential toward pathogenic microbes (Prylutsky et al. 2014). Cation-substituted fullerenes with quaternary amino groups have been shown to be effective against broad-spectrum pathogenic bacteria like *S. aureus*, *E. coli*, and *Candida albicans* (Mizuno et al. 2015). In another study, fullerene derivatives have been prepared using protonated amine and were found to have antibacterial activity against resistant *E. coli* (Deryabin et al. 2014). Huang et al. (2014) fabricated fullerenes with two types of functionalities using decacationic side chain and the other with an extra deca-tertiary-amine side chain. The decacationic side chain-containing fullerenes were potent broad-spectrum agents for antibacterial activity against both Gram-positive and Gram-negative bacteria. Similarly, a powerful bactericidal fullerene was created by using iodide groups which converted into I or I₂ upon photoelectron reduction leading to the generation of reactive oxygen species that caused damage to the bacteria (Zhang et al. 2015).

(iii) Modifications of Graphene Nanostructures

Graphene type of nanostructures includes graphene oxide nanoparticles, nanosheets, multilayer graphene, and pristine graphene (Al-Jumaili et al. 2017). Several graphene-containing nanostructures contain oxygen groups which make their surface negatively charged; hence, for a better interaction of graphene nanoparticles with the bacterial membrane, these are functionalized with mostly positively charged groups (de Faria et al. 2014). A recent study was conducted where graphene monolayer was wrapped on silver nanowires and it was observed that this particular modification led to efficient killing of the microbes (Li et al. 2016).

Graphene oxide nanoparticles have been considered as the new-generation nanoweapon against multidrug-resistant bacteria (Yousefi et al. 2017). Functionalization of graphene oxide nanoparticles with polymeric materials (such as dextran, chitosan, polyacrylic acid (PAA), poly-L-lysine (PLL), proteins, etc.) or linking with inorganic nanostructures to form nanohybrids (such as GO–Au, GO–Ag, GO–ZnO, GO–TiO₂ nanohybrids, etc.) has led to increased antibacterial efficiency of graphene oxide nanoparticles (Yousefi et al. 2017).

Li et al. (2016) synthesized graphene oxide nanoparticles with hydroxyl, oxidative, and carbon radical-containing groups and it was found that carbon radicals containing graphene oxide exhibited maximum bactericidal activity. On similar

lines, the bactericidal activity of graphene nanosheets after functionalizing them with silver has also been studied (Ma et al. 2011). The silver-functionalized graphene nanoparticles had significant antibacterial activity as compared to the activity of silver and graphene nanoparticles alone. Likewise, graphene oxide nanoparticles coated with gold have been found to inhibit a broad range of both Gram-positive and Gram-negative bacteria with 100% efficiency (Hussain et al. 2014). Graphene oxide nanoparticles have also been attempted to be modified by silane ligands for their attachment to silver ions. These nanocomposites showed potent antibacterial activities against *E. coli* and *S. aureus* pathogens (Fathalipour and Mardi 2017).

(iv) Modifications of Dendrimers

Dendrimers are basically nanoscaled molecules with a radial symmetry. These comprise a symmetrical core along with an inner and outer shell. The surface modification of dendrimers is performed to enhance their antibacterial activity towards many antibiotic-resistant strains. Their antimicrobial activities depend on the size and the nature of the attached functional group (Lind et al. 2015). For example, polyamidoamine dendrimers were found to hold higher antibacterial activity when this group was substituted with OH or COOH (Xue et al. 2015). Recent studies show the development of dendrimeric peptides for the control of multidrug-resistant strains having antibacterial, antifungal, and antiviral properties (Scorciapino et al. 2017). Phloroglucinol succinic acid dendrimers have also been synthesized with anionic surfaces. Out of these some of the dendrimers were found to be quite potent against *S. aureus*, *E. coli*, and human pathogen *C. albicans* (Kumar et al. 2015). Choi et al. (2012) reported the potential use of a dendrimer-based vancomycin nanoplateform as a targeting agent for a drug-resistant bacterial surface.

(v) Nanoparticle–Antimicrobial Peptide Conjugates

Antimicrobial peptides are potent antimicrobial agents produced by bacteria and eukaryotes, and these peptides have been proven to be more potent than the conventional antibiotics used for controlling the spread of multidrug-resistant strains (Nguyen et al. 2011). These antimicrobial peptides are broad-spectrum agents and attack both Gram-positive and Gram-negative strains. Despite their potential activity, the antimicrobial peptides, however, suffer from being employed in therapeutics due to their impermeability and low enzyme stability. Hence, the recent approaches to increase the efficacy of antimicrobial peptides include their grafting on different types of nanoparticles. While the antibacterial properties of nanoparticles have been well established, their conjugation with antimicrobial peptides has led to the development of a new and advanced technology in preventing the spread of antimicrobial resistance.

In the recent past, several studies on the potential role of antimicrobial peptide–nanoparticle conjugates as antimicrobial agents against drug-resistant pathogens have been documented. Of these, the most widely explored are antimicrobial peptide-conjugated graphene oxide nanoparticles (Rajchakit and Sarojini 2017). For example, in a recent study, gold nanoparticles conjugated with antimicrobial peptides, esculentin-1a(1–21)NH₂, have shown promising results against the

biofilm-forming Gram-negative pathogenic strain of *Pseudomonas aeruginosa* (Casciaro et al. 2017). Similarly, antimicrobial peptide–graphene oxide nanoparticle–DNA aptamer conjugates were developed as highly effective antibacterial therapeutics against *Vibrio vulnificus* (Lee et al. 2017). Other than graphene oxide nanoparticles, the conjugation of antimicrobial peptide, Clavanin A, with Fe_3O_4 –silane nanoshells for antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* has also been reported (Ribeiro et al. 2018). Conjugation of silver nanoparticles with Ubiquicidin (29–41), an antimicrobial peptide, demonstrated significant antibacterial effects against *E. coli* and *P. aeruginosa* (Morales-Avila et al. 2017).

(vi) Nanoparticle–Antibiotic Conjugates

Nanoparticles have been conjugated with known antibiotics *via* covalent or non-covalent interactions. Such nanoparticle–antibiotic conjugates have reportedly enhanced antimicrobial activity against multidrug-resistant bacteria. It is understood that this antimicrobial strategy using antibiotic functionalized onto metal nanoparticles can have promising applications in the design of antimicrobial, antiviral, or anticancer therapies. Moreover, the approach is also considered useful for reviving old unresponsive drugs, the efficacy of which can be restored upon conjugation with nanoparticles (Shaikh et al. 2017). Table 6.5 shows some of the metal/metal oxide nanoparticles that have been conjugated with antibiotics such as ampicillin, vancomycin, ciprofloxacin, imipenem, etc. and their efficacy against resistant/multidrug-resistant bacteria. Since multidrug-resistant bacteria are known to have evolved methods for countering all classes of beta-lactam antibiotics, Brown et al. (2012) explored the efficacy of ampicillin molecules conjugated onto silver and graphene oxide nanoparticles against beta-lactam-resistant bacteria. Both graphene oxide nanoparticle–ampicillin and silver nanoparticle–ampicillin conjugates were found to exhibit potent bactericidal activity against multidrug-resistant bacteria. Similarly, ampicillin was conjugated onto lysozyme-capped graphene oxide nanoclusters (AUNC-L-Amp) to form a “broad-spectrum antibacterial hybrid” (Kalita et al. 2018). The system showed reversion of resistance by methicillin-resistant *S. aureus* and increased inhibitory effect. This was attributed to multiple reasons such as increased ampicillin concentration at antibiotic–bacteria interaction site, multivalent presentation of ampicillin, enhanced permeation of the antibiotic through lysozyme-mediated cell wall lysis, or graphene oxide ion-induced cell destabilization. Capeletti et al. (2014) demonstrated the encapsulation of tetracycline into silica nanoparticles and their high bactericidal effects against multiple resistant *E. coli*. A study conducted on titanium/titanium oxide nanoparticles also showed the enhancement in their bactericidal effect against *S. aureus* when they were functionalized with ampicillin (Pissinis et al. 2018).

(vii) Effect of Quantum Dots

Quantum dots are a unique class of semiconductors, with size ranging from 2–10 nm. Very recently, quantum dots have also been investigated for their effectiveness in combating superbugs. Courtney et al. (2016) showed that

Table 6.5 Effect of nanoparticle–antibiotic conjugates on drug-resistant bacteria

Nanoparticle	Nanoparticle size and shape	Resistant bacteria	Observation	Reference
Magnetic nanoparticles covalently linked to vancomycin	3–4 nm	Vancomycin-resistant <i>Enterococci</i>	Captures vancomycin-resistant <i>Enterococci</i> and other Gram-positive bacteria at ultralow concentrations	Gu et al. (2003)
Ampicillin-functionalized gold and silver nanoparticles	Spherical, silver nanoparticle: 4 nm; graphene oxide nanoparticle: 7 nm	Multidrug-resistant <i>P. aeruginosa</i> , <i>E. aerogenes</i> , and methicillin-resistant isolate of <i>S. aureus</i>	Gold and silver nanoparticles killed multidrug-resistant <i>P. aeruginosa</i> , <i>E. aerogenes</i> , and methicillin-resistant <i>S. aureus</i>	Brown et al. (2012)
			Killing capacity of silver nanoparticle increased upon functionalization with ampicillin; killed ampicillin-resistant bacteria faster than silver nanoparticle	
Zinc oxide nanoparticles functionalized with ciprofloxacin	Spherical, 18–20 nm	Multidrug-resistant <i>E. coli</i> , <i>S. aureus</i> , and <i>Klebsiella</i> sp.	Excellent antibacterial activity exhibited	Patra et al. (2014)
Imipenem-functionalized iron oxide (Fe ₂ O ₃) nanoparticles	20–50 nm	<i>P. aeruginosa</i> ATCC: 27853	Excellent antibacterial effects observed compared to Fe ₂ O ₃ nanoparticles	Khataminejad et al. (2015)
			Synergistic effect with antibiotics in counteracting with bacterial resistance	
Silver-silica core-shell nanoparticles functionalized with ampicillin	Spherical, 93 nm	Ampicillin-resistant <i>E. coli</i>	100% inhibition of bacterial growth	de Oliveira et al. (2017)
			50% reduction in number of colonies for ampicillin-resistant <i>E. coli</i>	
			No toxicity to mammalian cells	

(continued)

Table 6.5 (continued)

Nanoparticle	Nanoparticle size and shape	Resistant bacteria	Observation	Reference
Cefotaxime-conjugated gold nanoparticles	Gold nanoparticle: 6.87–2.43 nm; cefotaxime-conjugated gold nanoparticle: Spherical, 17.55–2.95 nm	Extended-spectrum beta-lactamase (CTXM-15)-positive cefotaxime-resistant <i>E. coli</i> and <i>K. pneumoniae</i>	Antibiotic–nanoparticle conjugate showed bactericidal effect on both bacteria which were completely resistant to cefotaxime	Shaikh et al. (2017)
Lysozyme-capped gold nanoclusters functionalized with ampicillin	Spherical, 1–5 nm	Methicillin-resistant <i>S. aureus</i>	Reverted the Methicillin-resistant <i>S. aureus</i> resistance toward ampicillin	Kalita et al. (2018)
			Demonstrated enhanced antibacterial activity against nonresistant bacterial strains	
Silver functionalized by thiosemicarbazide with ciprofloxacin	–	Ciprofloxacin-resistant <i>P. aeruginosa</i>	Efficiently inhibited bacterial growth	Abdolhosseini et al. (2019)
			Reduced the expression of efflux pump genes	
Silver nanoparticles with ampicillin	Spherical 10 nm	Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	Ampicillin-coated silver nanoparticles helped in inhibiting the growth of bacteria thus limiting the infections caused by them	Surwade et al. (2019)
Nanopolymersomes made of silver-containing methicillin	Spherical diameter ranging 40–300 nm	Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	Bacteriostatic activity and nontoxicity toward human cell lines	Bassous and Webster (2019)

photoexcited quantum dots could kill a wide range of multidrug-resistant bacterial clinical isolates, including methicillin-resistant *S. aureus*, carbapenem-resistant *E. coli*, and extended-spectrum β -lactamase-producing *K. pneumoniae* and *S. typhimurium*. Quantum dots were able to kill 92% of bacterial cells in monoculture, as well as in a co-culture of *E. coli* and HEK 293 T cells, without affecting other mammalian cells. In another study, the same research group showed that light-activated, superoxide-producing cadmium telluride quantum dots could enhance the activity of antibiotics against clinical multidrug-resistant isolates of *E. coli*, *S. enterica*, and *K. pneumoniae* through quantum dot–antibiotic synergistic

interaction (Courtney et al. 2017). The quantum dots system was found to be thousand times more effective in killing bacteria over single use of antibiotic. Exposure of methicillin-resistant *S. aureus* and *E. coli* to photoexcited graphene quantum dots led to bacterial killing due to reactive oxygen species generation (Ristic et al. 2014). Quantum dots have also been modified for enhanced antimicrobial properties. In one such study, Kuo et al. (2018) showed graphene quantum dots, when doped with nitrogen and functionalized with an amino group, exhibited improved reactive oxygen species generation ability compared to unmodified graphene quantum dots, and completely eliminated multidrug-resistant bacteria.

6.5 Conclusions and Future Perspectives

The rise in the emergence of antibiotic-resistant strains has led to an urgent requirement of efficient solutions to combat their fast growth. With nanomaterials emerging as prominent antibacterial agents, nanotechnology, undoubtedly, holds great hope toward combating multidrug-resistant strains and benefitting public health. Apart from antimicrobial effects of metal and metal oxide nanoparticles, new approaches to enhance the bactericidal effect of nanoparticles such as use of carbon nanotubes, fullerenes, dendrimers, antimicrobial peptides, etc. have also been materialized in recent years. While the modifications of nanoparticles have led to improvement in the control of antibiotic-resistant strains, the *in vivo* and *in vitro* behavioral characteristics of these modified nanostructures need to be explored upon. Also with the introduction of nanoantibiotics the future holds tremendous scope for the development and delivery of novel antibiotic formulations for treating many resistant strains.

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Chapter 7

Toxicity of Engineered Nanostructures in Aquatic Environments



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Abstract The importance of engineered nanostructures has consistently increased along the last years due to the large number of applications intended for those materials, ranging from drug delivery systems, nanoelectronics and nanophotonics, and smart food packaging, to cite a few. However, the extensive use of such nanomaterials, if not properly employed and disposed, raises concern on the toxicity they can present to humans and aquatic organisms. The latter, specifically, compose the basic

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trophic levels for many other organisms, including terrestrial ones, and therefore, toxicity aspects of nanoengineered nanostructures play a critical role. In this chapter, we review some important nanostructures, including carbonaceous materials as carbon nanotubes and graphene, and also varied nanoparticles, including copper oxide, hydroxyapatite, silver, and zinc oxide nanoparticles, as well as their toxicities toward aquatic organisms, including microalgae, microcrustaceans, and fishes. We also present some aspects about nanostructure risk assessments for nanomaterials, and, finally, we show some trends regarding toxicity aspects and protection regulation.

Keywords Toxicity tests · Ecotoxicology · Algae · Daphnia · Fish · Graphene · Silver nanoparticles · Carbon nanotubes · Hydroxyapatite

7.1 Introduction to Nanomaterials and Toxicity Aspects

Nanotechnology has allowed the development of engineered nanostructures, including quantum dots, nanoparticles, nanofibers, nanostructured films, etc., with customized properties for varied applications. Such nanostructures display a high surface area/volume ratio (and consequently higher chemical reactivity) and quantum confinement effects, which allow them with special features to be used in applications ranging from nanoelectronics and nanophotonics, drug delivery systems, tissue engineering platforms, smart food packaging nanocomposites, sensors and biosensors, etc. (Mauter et al. 2018; Andre et al. 2018; Mercante et al. 2017; dos Santos et al. 2020).

In agriculture, nanotechnology has been applied on the development of novel fertilizers, herbicides, pesticides, and veterinary drugs (Prasad et al. 2014; Rai et al. 2014; FAO/WHO 2010) with the objective of optimizing their release in a controlled manner so that product delivery occurs selectively, with minimum chemicals utilization. For instance, nanoencapsulation has been successfully employed as a more efficient and safer strategy for handling pesticides with less harm to the environment (Nair et al. 2010; Rai et al. 2014).

Engineered nanostructures are of pronounced significance due to the large possibilities of chemical synthesis routes and functionalization for improving the material's final properties. Traditional nanostructures include silver nanoparticles, graphene oxide, zinc oxide nanoparticles, titanium dioxide nanoparticles, and single-walled or multiwalled carbon nanotubes. Silver nanoparticles, for instance, are nowadays widely employed in commercialized products, mainly due to their remarkable antibacterial properties (McGillicuddy et al. 2017).

However, the widespread use of engineered nanostructures in food industry and agriculture raises concern about toxicological effects to the humans and biota. For instance, nanomaterials can reach the aquatic environment, through several routes, including surface leaching, municipal wastewater treatment plants, and water purification purposes. That is the reason why aquatic ecosystems can be considered as

the major sink of anthropogenic contaminants. Thus, investigations focusing on the route of nanomaterials release and their effects on the aquatic biota are of paramount importance (Griffitt et al. 2012) for risk assessment purposes.

Considering the various nanomaterials' physicochemical properties and abiotic factors that influence their ecotoxicity, both spatially and over time, there is still a gap of knowledge between nanotoxicological research and nanomaterial safety that must be accomplished. There is variability in toxic effects of nanomaterials that depends on the aquatic organism, exposition time, and route used to prepare the materials. Bioavailability is influenced by water quality parameters and nanomaterial formulation. Once available in the aquatic environment, nanomaterials can be affected by their surroundings and undergo transformations by three major phenomena: dissolution, organism-dependent cellular uptake, and promotion of oxidative stress and consequent cellular damages (Ivask et al. 2014). Reactive oxygen species (ROS) can induce oxidative stress resulting in cyto- and genotoxicity and trigger the induction of antioxidant enzymes that are used as response biomarkers (Vale et al. 2016). Although oxidative stress can be a driver for many nanoparticle-induced effects, nanoparticles have the ability to act via multiple pathways (Bundschuh et al. 2018).

The dispersion stability of nanomaterials can influence their environmental fate by defining dissolution rate/surface reactivity, effective size, and long-range transport (Monikh et al. 2018). Surface-functionalized particles can limit or inhibit environmental transformations, which influence particle aggregation, mobility, dissolution, and ecotoxic potential (Shevlin et al. 2018). In addition, contaminants as micro-pollutants with different chemical properties can interact with several types of nanoparticles. For example, carbon nanomaterials can synergistically and/or antagonistically interact with these contaminants.

The consideration of the effect assessment at different trophic levels is necessary in order to evaluate with more accuracy the probability of adverse effects and to determine safe concentrations in the aquatic compartments (USEPA 1994). In order to establish such concentration limits, toxicity studies in at least three trophic levels are necessary: one primary producer (e.g., algae), one primary consumer (e.g., microcrustaceans), and one secondary consumer (e.g., fish) (European Commission 2003), which are important sources of food for larger animals (Suthers and Rissik 2009). The species-sensitivity distribution (SSD) method can provide a reliable statistics for the determinations of safe concentrations of nanomaterials. This approach is based on an recognized distribution of a toxicity data set obtained from different species, including algae and invertebrates among other aquatic organisms (Lei et al. 2012; Garner et al. 2015; Castro et al. 2018). In this context, this book chapter reviews on some syntheses methods employed for producing varied engineered nanoparticles, including silver nanoparticles, carbon nanotubes, zinc oxide nanoparticles, copper oxide, graphene-based materials, and hydroxyapatite nanoparticles and illustrates some examples of how the shape and functionalization of such nanoparticles can influence their toxicity toward microalgae, microcrustaceans, and fishes.

7.2 Engineered Nanostructures: Synthesis Methods

7.2.1 Carbon Nanotubes

Carbon nanotubes are nanomaterials with enormous potential applications in fields such as medicine, automobile and aviation industries, energy, defense, and diagnosis applications (Prasek et al. 2011; Eatemadi et al. 2014; Li and Zhang 2015; Stout 2015; Zhai et al. 2016; Yadav et al. 2017; Hussain et al. 2018). Carbon materials can have different constructed structures with entirely different properties (Eatemadi et al. 2014). In carbon nanotubes, carbon presents a sp^2 hybridization, with weak out-of-plane bonding and strong in-plane bounds (Yadav et al. 2017). Carbon nanotubes can be obtained as fibers, films, yarns, ropes, single-walled carbon nanotubes (SWCNTs), and also multiwalled carbon nanotubes (MWCNTs).

Several methods can be employed for obtaining carbon nanotubes, and most of them rely on gas-phase processes (Prasek et al. 2011; Journet et al. 2012; Eatemadi et al. 2014; Yadav et al. 2017). Some of the currently employed methods for producing carbon nanotubes are illustrated in Fig. 7.1 (Prasek et al. 2011), and also in other references available in the literature (Journet et al. 2012).

In general, the carbon nanotube syntheses are classified according to the temperature (high, medium, and low) employed. The high-temperature methods are subdivided in electric arc discharge, laser ablation, and vaporization induced by a solar beam. The medium-temperature methods include in situ catalysts on gas phase and synthesis by supported catalysts. Finally, the low-temperature methods include plasma-enhanced CCVD (PE-CCVD) and laser-assisted catalytic chemical vapor deposition (LA-CCVD) (Journet et al. 2012).

Perhaps the most traditional techniques for producing SWCNTs and MWCNTs are the carbon arc-discharge technique (Ando and Iijima 1993; Journet et al. 1997; Zhao et al. 1997; Shi et al. 1999), laser ablation technique (Zhang et al. 1998; Zhang and Iijima 1999; Scott et al. 2001), and chemical vapor deposition (CVD) technique (Kong et al. 1998; Colomer et al. 2000; Hofmann et al. 2003; Kumar and Ando 2010). Although SWCNTs and MWCNTs can be synthesized without transition metal catalysts, the use of catalysts (such as Ni or Co) can lead to a better control of carbon nanotube properties, including diameter and chirality, however with the disadvantage of increasing costs (Yadav et al. 2017). Below, we present in more details some of these techniques employed for producing carbon nanotubes.

The carbon arc-discharge technique: it consists of vaporizing carbon in the existence of catalysts (cobalt, iron, nickel, etc.) under a reduced atmosphere (inert gas as helium or argon). After the arc-discharge is formed between two electrodes, a plasma composed of carbon vapor, inert gas, and the catalysts vapor is formed. The vaporization arises as a consequence of the energy transferred from the arc to the anode made of graphite doped with catalysts. A considerable amount of impurities and/or amorphous carbon can be obtained through it.

Laser ablation technique: this is another technique for producing carbon nanotubes, which employs a high-power laser beam to vaporize the carbon from either a



Fig. 7.1 Examples of distinct methods for producing CNTs. (Reprinted from Prasek et al. 2011 with permission of The Royal Society of Chemistry)

graphite target at high temperature or a gas containing methane or carbon dioxide, resulting in the formation of pristine carbon nanotubes.

Chemical vapor deposition (CVD): this technique is usually applied for producing carbon nanotubes in scalable industrial production due to its mild operations and low cost. This technique has two main approaches: floating catalyst chemical vapor deposition (FCCVD) and fixed catalyst chemical vapor deposition (Prasek et al. 2011; Eatemadi et al. 2014; Yadav et al. 2017).

7.2.2 Copper Nanoparticles

Copper oxide nanoparticles present remarkable physical and chemical properties and have been used for different applications, including bactericide materials, sensors, catalysts, conductive films, lubrication, nanofluids, semiconductors, batteries,

gas sensors, biosensors, and field transistors (Yang and He 2011; Longano et al. 2012; Harikumar and Aravind 2016; Prabhu et al. 2017). The synthesis control of copper oxide micro- and nanostructures is responsible for the development of new applications and the improvement of the existing ones, once one can tailor their morphological, chemical, and physical properties. Currently, copper oxide nanoparticles can be obtained in distinct shapes and sizes, including nanorods, nanowires, nanotubes, nanoplatelets, nanodendrites, and nanoflowers (Vijaya Kumar et al. 2001; Cao et al. 2003; Narayanan and El-Sayed 2003; Li et al. 2004; Li et al. 2005; Song et al. 2007).

Copper nanomaterials can be obtained by distinct chemical, physical, and biological methods, yielding nanoparticles with different morphologies, sizes, and chemical and physical properties (Zhang et al. 2014). The synthesis methods include metal vapor synthesis (MVS), exploding wire method, vacuum vapor deposition, sonochemical reduction, thermal reduction, chemical reduction, biosynthesis, laser irradiation, microemulsion techniques, sol–gel technique, thermal oxidation method among many others (Song et al. 2007; Naika et al. 2015; Suárez-Cerda et al. 2017). Next we briefly describe some of the methods.

Metal vapor synthesis (MVS): it consists of the sublimation and posterior recondensation of a metal under a high vacuum, aiming at combining its atoms or small particles with ligands, preparing metal complexes. Normally, a reactor is used to evaporate the metal, and the resulting vapor then collides with a cold wall containing the organic ligand. This method allows the production of small and homogeneous metal nanoparticles without using reactants during the nanoparticle production (Barbaro et al. 2015).

Exploding wire method: in this method, nanopowder is produced by electric explosion of metallic wire in an inert gas. The process starts with the application of a rising current to the metallic wire, in which high heat generated makes the wire to vaporize and create an electric arc, which is the responsible of the explosion. The average particle size, its properties, and chemical composition can be determined using different parameters, such as the voltage of the capacitor used to generate the current, which leads to a wide variety of products (Tarasov et al. 2002).

Vacuum vapor decomposition (VDD): this method is usually employed for producing copper nanorods and nanowires, under very low pressure or vacuum conditions, where copper vapor is generated and then re-deposited on a specific target/substrate. VDD was proposed by Liu and Bando (2003), where they used a tiny copper ring inside a dry pumping station with a low pressure to generate the vapor and a molybdenum grid as the substrate. The diameter of the copper nanorods formed varied from 50 to 100 nm.

Sonochemical reduction: Vijaya Kumar et al. (2001) showed that it is possible to synthesize amorphous Cu and nanocrystalline Cu₂O using an ultrasonic horn (Ti-horn, 20 kHz, 100 W cm⁻²) and aniline as a solvent. With the methodology proposed, the authors have obtained Cu particles from 4 to 10 nm and Cu₂O particles from 5 to 13 nm, with a suitable distribution within the polyaniline matrix.

Chemical reduction: this method is perhaps the most popular synthesis method for obtaining Cu nanoparticles (Suárez-Cerda et al. 2017), due to the easy control of

nanoparticles size and shape, besides being a simple methodology. On the other hand, it can be harmful to the environment, because of the use of toxic solvents and chemical reducing agents like sodium borohydride and diethylenetriamine, among others. In this method, typically the reduction of metal salts is performed in a solvent and a separate reducing agent (Dang et al. 2011).

Solution-based methods: these are common and effective methods to obtain copper oxide nanoparticles with controllable shape, composition, and reproducibility (Zhang et al. 2014). The reaction usually has low temperature and large-scale production, and synthesis parameters control the compositions, sizes, and dimensions of the copper oxide nanostructures. Hydrothermal and chemical precipitation techniques are two of the most used techniques to synthesize copper oxide nanostructures.

Solid-state thermal conversion of precursors: this method is employed for obtaining copper oxide nanostructure by thermal conversion of precursors, and it is similar to the solution-based chemical precipitation method. However, the thermal dehydration of cupric precursors is accomplished in solid state using higher temperature (Zhang et al. 2014).

Electrochemical method: nanostructured transition metal oxides (MOs) or nanoporous MOs, including copper oxide nanostructure, can be prepared using electrochemical method because of its simplicity and the operation process that involves low temperature. Some advantages of this method include nanostructures with higher growth orientation and controlled morphology and size (Zhang et al. 2014).

Thermal oxidation method: it can be employed to synthesize 1D copper oxide nanostructures, which consists of heating Cu substrates in air, during which the reaction between Cu and oxygen (O_2) occurs. The parameters that can change the morphologies of copper oxide are the oxidation temperature, growth time, and others (Singh and Ali 2010; Li et al. 2012; Filipič and Cvelbar 2012).

Biosynthesis and green synthesis: currently, the use of “green” synthesis has also been pursued for producing nanomaterials with less environmental impact, where the nanoparticles can be recovered from natural resources, for example, from microorganisms and plants (Kulkarni and Muddapur 2014), such as neem, alfalfa, *Cinnamomum camphora*, *Emblica officinalis*, lemon grass, tamarind, and *Euphorbia tirucalli* (Naika et al. 2015).

7.2.3 Graphene

Graphene is composed by sheets of carbon atoms possessing sp^2 hybridization, presenting two-dimensional hexagonal structure with a high surface area (Novoselov et al. 2004; Nogueira et al. 2015). They possess remarkable thermal, electronic, and mechanical properties finding applications in the design of supercapacitors, nanoelectronics, nanosensors, and drug delivery systems (Facure et al. 2020; Novoselov et al. 2004; Pérez et al. 2009; Wang 2011; Baptista-Pires et al. 2014; Liu et al. 2016; Andre et al. 2017; Mercante et al. 2017). Owing to the widespread use of

graphene-based materials including graphene oxide and reduced graphene oxide, concerns have been raised about the nanotoxicological effects that they can cause in the environment, reaching all trophic levels, from aquatic through terrestrial ecosystems (Ou et al. 2016; Guo et al. 2017; He et al. 2017; Zhang et al. 2017; Zhao et al. 2017; Garacci et al. 2017; Katsumiti et al. 2017).

Graphene can be obtained from crystalline graphite by different chemical routes (Ou et al. 2016). According to Paulchamy et al. (2015), the structure and properties of graphene oxide are dependent on the type of synthesis method and also on the degree of oxidation process. The types of graphene and their derivatives may include monolayer graphene, graphene (2–5 layers), multilayer graphene (2–10 layers), graphite nanoplates, graphene oxide, reduced graphene oxide, graphene nanosheets, graphene quantum dots, among others (Geim 2009; Park et al. 2009; Seabra et al. 2014; Guo and Mei 2014; Facure et al. 2020).

The syntheses methods for graphene oxide and reduced graphene oxide will be focused in this review, which are most studied in the literature. Figure 7.2a shows the monolayer graphene. Graphene oxide is shown in Fig. 7.2, being an

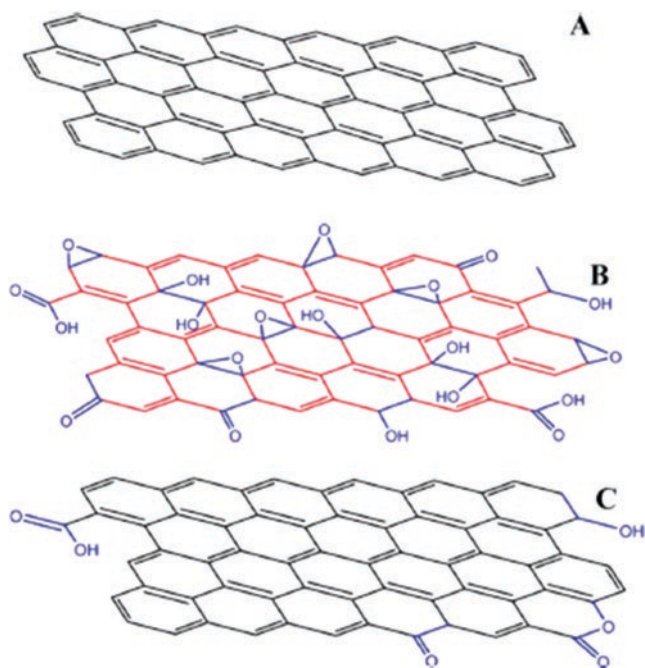


Fig. 7.2 Structural models of (a) monolayer graphene, (b) graphene oxide, and (c) reduced graphene oxide. Functional groups such as hydroxyl, carboxyl, and epoxy groups may be introduced into graphene oxide (b) after the oxidative exfoliation. For reduced graphene, the oxides (c), hydroxyl, and carboxyl groups can still remain on the edge of graphitic sheets due to incomplete reduction. (Figure adapted from Zhao et al. 2014, reprinted with permission from American Chemical Society)

intermediate product of reduced graphene oxide synthesis (Fig. 7.2c) prepared by oxidative exfoliation of graphite (Loh et al. 2010; Dreyer et al. 2010).

All the synthesis methods involve the oxidation of graphite. The synthesis method by Hummers uses a broad variation of oxidizing agents to exfoliate graphite flakes. The Brodie and Staudenmaier methods combine a mixing of potassium chlorate (KClO_3) with nitric acid (HNO_3) to induce oxidation of graphite. In contrast, Hummers method uses potassium permanganate (KMnO_4) and sulfuric acid (H_2SO_4) (Hummers and Offeman 1958; Tang et al. 2013; Shahriary and Athawale 2014; Nanda et al. 2015; Liu et al. 2016).

According to the synthesis methods for obtaining reduced graphene oxide from graphene oxide, sodium citrate is used as a reducing agent. Sodium citrate is mixed with an aqueous dispersion of graphene oxide and maintained for 24 h under reflux, until the dispersion color changes from light brown to homogeneous black color. The excess of sodium citrate is removed by centrifugation, and the resulting precipitate can be redispersed in water (Zhang et al. 2011).

7.2.4 Hydroxyapatite Nanoparticles

Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a natural bioceramic material which presents potential applications in areas ranging from fertilizers, drug delivery systems, pharmaceuticals, water treatment, and biomedicine (Geng et al. 2017; Haider et al. 2017; Pereira et al. 2017; Wijesinghe et al. 2017). There are several methods by which nanoparticles can be obtained, including hydroxyapatite (Haider et al. 2017). For instance, hydroxyapatite nanoparticles can be obtained by several methods, and a selection of them is displayed below.

Wet Synthesis

Conventional homogeneous wet-chemical precipitation: this method is widely used due to its simplicity. The hydroxyapatite nanoparticles are synthesized by mixing the aqueous solutions containing the orthophosphate and calcium, which are then submitted to specific temperature and pressure in order to obtain the stoichiometry, crystalline phase, and grain size desired. Results available in the literature show that the reaction speed is slow, the crystallinity is poor, and the control of the growth is hard (Jarcho et al. 1976; Ahn et al. 2001; De Lima Souza et al. 2008).

Hydrothermal treatment: this method consists of the reaction of an aqueous solution of calcium and phosphorous precursors at ambient pressure and temperature. When compared with others methods, hydrothermal treatment is a suitable strategy to obtain hydroxyapatite nanoparticles with high crystallinity and dispersibility (Okada and Furuzono 2012; Qi et al. 2017), but may present difficulties for controlling the size distribution and morphology (Sadat-Shojai et al. 2013).

Dry Methods

Solid-state synthesis: this synthesis method consists of the use of the precursors that are first milled and then calcinated at a high temperature (typically above 700 °C), leading to the formation of a well-crystallized structure (Monmaturapoj and Yatongchai 2010; Qi et al. 2017). The precursors can be, for example, CaCO_3 and CaHPO_4 .

Molten salt synthesis: it employs a molten salt for preparing complex oxides from their constituent materials as oxides and carbonates. This method allows synthesizing hydroxyapatite nanoparticles with high thermal stability but having the inconvenience of employing high temperatures (>800 °C) (Taş 2004; Sadat-Shojai et al. 2013; Qi et al. 2017).

7.2.5 Silver Nanoparticles

There are several methods available for synthesizing silver nanoparticles (Silva et al. 2017a), which can be divided into chemical, physical, and biological methods (Silva et al. 2017a; Ahmed et al. 2017) Tran et al. 2013), which employ bottom-up and top-down approaches (Tolaymat et al. 2010; Abou El-Nour et al. 2010; Silva et al. 2017a; Ahmed et al. 2017). Bottom-up approaches are those based on chemical methods that make silver nanoparticles from silver atoms (Tolaymat et al. 2010). On the other hand, top-down approaches are mainly physical methods that employ mechanical forces to downgrade macroscopic silver to the nanoscale size (Ju-Nam and Lead 2008). More details on some of the synthesis methods are given below.

Chemical approach: this is the most common method used for obtaining silver nanoparticles (Sharma et al. 2009; Tolaymat et al. 2010). Chemical silver nanoparticles can be synthesized by basic elements/ingredients, including metal salt precursors, solvents, reductants, and stabilizing agents (Tolaymat et al. 2010; Tran et al. 2013). In this method, ionic silver is reduced and then precipitated to form the nanoparticles (Rai et al. 2009; Tran et al. 2013). Silver nitrate is the most used salt precursor (Tolaymat et al. 2010), owing to the chemical stability and low cost, followed by silver acetate, silver perchlorate, silver sulfate, and others of low use. Water is used as solvent in about 80% of the synthesis processes, followed by ethanol, dimethylformamide (DMF), EG, toluene, and others of less importance (Tolaymat et al. 2010). Reductants are important for providing free electrons to reduce silver ions and form nanoparticles (Tolaymat et al. 2010). The most used reductant agents are borohydride and citrate, and others such as ascorbate and elemental hydrogen (Tolaymat et al. 2010; Abou El-Nour et al. 2010; Becaro et al. 2015; Ahmed et al. 2017, Trans et al. 2013). Stabilizing agents are used to prevent particles from aggregation and to yield nanoparticle of uniform size. The most used stabilizing agents for silver nanoparticle synthesis are citrate, PVP, amines, amide, and others (Tolaymat et al. 2010).

Physical approach: this method involves mechanical approaches such as grinding, milling, and thermal/laser ablation (Ahmed et al. 2016) to produce the nanoparticles. Other physical methods include thermal decomposition and evaporation–condensation with tube furnace at atmospheric pressure. However, the latter has a number of disadvantages, such as the room necessary for the equipment, energy consumption, and temperature stability (Abou El-Nour et al. 2010).

Biological and green syntheses: more recently, biological and green syntheses (Ahmed et al. 2016; Tran et al. 2013) of silver nanoparticles have gained some popularity (Silva et al. 2017a). Silver nanoparticles obtained by biological synthesis are those produced by living organisms (Tran et al. 2013), such as bacteria (e.g., *Bacillus* sp and *Lactobacillus*), fungi, yeasts, or algae (Kaler et al. 2010).

Plant extracts such as *Cassia angustifolia* and *Daucus carota* (Kaler et al. 2010; Tran et al. 2013) have been studied, and many authors consider that such synthesis is environmental friendly due to the high rate of metal ion reduction (Ramya and Subapriya 2012; Tran et al. 2013). However, contaminants and restrictions on replying results can be considered as a limitation (Kumar and Yadav 2009; Kumar et al. 2010; Kumar and Yadav 2012; Silva et al. 2017b).

7.2.6 Zinc Oxide Nanoparticles

Mechanochemical processing (MCP) and physical vapor synthesis (PVS) are two widely used methods employed for obtaining zinc oxide nanoparticles (Espitia et al. 2012), but other synthetic methods can be considered, such as precipitation, thermal decomposition, and hydrothermal synthesis. MCP combines physical and chemical methods, where the particles are reduced mechanically and chemically during milling. It is a simple and low-cost method and does not involve organic solvents, being less harmful to environment (Lu et al. 2008; Espitia et al. 2012). Physical vapor synthesis (PVS) consists of the application of plasma arc energy under high temperature on contact with a solid precursor, requiring high energy to produce the reactions (Moezzi et al. 2012; Espitia et al. 2012). Chemical methods that are also employed for obtaining zinc oxide nanoparticles include microemulsion, Sol–gel method, precipitation, chemical reduction, hydrothermal method, solvothermal method, and chemical vapor deposition. More recently, biological methods have become an important methodology for obtaining zinc oxide nanoparticles (Dobrucka and Długaszewska 2016; Ahmed et al. 2017). However, biological methods involve a number of steps, as the selection of the organisms is more adapted to this function and appropriate conditions, which hinders industrial applications.

7.3 Toxicity of Engineered Nanostructures in Aquatic Environments

7.3.1 Nanotoxicity Investigations in Microalgae and Microcrustaceans

Toxicity Assays with *Pseudokirchneriella subcapitata*

Among unicellular green algae used in the risk assessment of nanomaterials and others chemicals agents (Li et al. 2016), the microalgae *Pseudokirchneriella subcapitata* (*Chlorococcales*, *ex-Selenastrum capricornutum*) is one of the most used standard organisms in ecotoxicological evaluation (Jonsson and Aoyama 2009; Machado and Soares 2014) because of its rapid reproduction and cosmopolitan distribution (Meena 2017; Oropesa et al. 2017).

The researchers usually grow this organism in culture medium in accordance with the methodology recommended by the Organization for Economic Co-operation and Development (OECD 1984, 2006) and United States Environmental Protection Agency (USEPA 2002). For the tests, a control (without the test material) and at least five concentrations of the nanomaterial, arranged in a geometric series, are selected for the definitive test. The cell density in the inoculum is determined by absorbance measurements at 750 or 684 nm in a spectrophotometer or in a microplate reader, once absorbance is proportional to the cell concentration. These determinations are conducted at 0, 24, 48, 72, and 96 h, but may be extended for 7 days. Data are fitted using a linear regression model that generates the angular coefficient, which gives algae growth rate, expressed as logarithm of absorbance per unit time. The calculation of the concentration capable of inhibiting algal growth rate by 50% on a given period (EC50) relies on the relative inhibition of growth rate as a function of the nanomaterial concentration (Castro et al. 2018; OECD 1984).

The adverse effects of graphene oxide on the growth rate of *P. subcapitata* were evaluated giving an EC50-72h equal to 66.60 (60.80–78.43) mg/L (Castro et al. 2018). This value is different from that calculated by Aruoja et al. (2009) for particulate nanomaterials like nano TiO₂ (EC50-72h = 5.83 mg Ti/L) and nano CuO (EC50-72h = 0.71/ mg Cu/L) to the same algae, indicating a lower toxicity of large carbon nanostructures than metals nanoparticles. Furthermore, the last authors showed that the metal toxicity was enhanced in several times when in nanoformulation, comparatively to the bulk formulation (bulk TiO₂ EC50-72h = 35.0 mg Ti/L and bulk CuO EC50-72h = 11.55 mg Cu/L).

Polymeric nanoparticles have potential applications in the development of nanocarriers for delivering active compounds like drugs and pesticides (Lopes et al. 2014; Oliveira et al. 2018). Therefore, polymeric nanoparticles can be used to provide effective pesticide formulations which are less toxic to nontarget organisms (Chauhan et al. 2017). In this context, Table 7.1 shows the results of a study in which chitosan nanoparticles were loaded with the herbicide paraquat and added to *P. subcapitata* cultures (Grillo et al. 2015). The influence of the aquatic humic

Table 7.1 Growth inhibition of *P. subcapitata* cultures exposed to chitosan nanoparticles, paraquat, and their associations with humic substances

	EC50-96h (mg/L)	Lower limit	Upper limit
Nanoparticles	>50.0 ^a	–	–
Paraquat	0.48 ^b	0.24	0.77
Paraquat associated with the nanoparticles	1.15 ^c	1.04	1.32
Paraquat associated with humic acid	0.78 ^b	0.66	0.91
Nanoparticles with paraquat and humic acid	1.65 ^c	1.28	2.29

Adapted from Grillo et al. (2015)

^{a,b,c} Different letters indicate statistically significant ($p < 0.05$) differences between the groups. The 96-h CE50 values for different test groups are compared with respect to control at $p < 0.05$. Each letter indicates significance with respect to control at $p < 0.05$

Table 7.2 Toxicity of two herbicides, poly(epsilon-caprolactone) nanocapsules and their associations to the microalgae *P. subcapitata*

	EC50-96h (µg/L)	Lower limit	Upper limit
Atrazine	97.06	61.11	133.01
Ametrine	12.60	10.58	14.95
Nanocapsules with atrazine	274.91	220.90	333.82
Nanocapsules with ametrine	267.33	154.37	399.11
Nanocapsules	2410.23	2108.95	2815.65

Adapted from Clemente et al. (2013)

substances on the colloidal toxicity of the herbicide was evaluated. Results indicated that paraquat associated with nanoparticles significantly reduced its toxicity to algae. The CE50-96h value obtained for paraquat alone was highly toxic ($0.1\text{--}1\text{ mg L}^{-1}$), while that for paraquat associated to nanoparticles was moderately toxic ($1\text{--}10\text{ mg L}^{-1}$). The inclusion of humic substances did not cause significant changes in the CE50-96 h values, although the data implied a lower toxicity trend when the organisms were co-exposed to humic acid. Lastly, the association of paraquat nanoparticles could significantly reduce its toxicity to algae.

In another study, Clemente et al. (2013) investigated the effect of two triazine herbicides encapsulated in poly(epsilon-caprolactone) nanocapsules. The results are shown in Table 7.2 and demonstrate that the toxicity of the nanocapsules without herbicide was much lower than that for nanocapsules containing the herbicides. In analogy to that described before with paraquat, the free herbicides caused greater inhibition of the growth of the algae than the encapsulated herbicides.

The two studies described before indicate that the encapsulation of herbicides in nanomaterials is capable to reduce the toxic impact on non-target organisms and that the herbicides delivery systems have potential applications for agriculture.

Toxicity Assays with Microcrustaceans *Daphnia* and *Artemia*

Toxicity tests employing both aquatic arthropoda (*Daphnia* and *Artemia*) are widely used to evaluate the potential risk of environmental contamination by nanomaterials (Chen et al. 2017; Zhu et al. 2017; Novak et al. 2018; Johnson et al. 2017). These microcrustaceans represent the invertebrates at the base of the food chain, so toxic effects at this trophic level can compromise the entire ecosystem (Dorchin and Shanas 2013; Yang et al. 2018).

Daphnia sp is a bioindicator organism listed in the “Manual of tests for evaluation of ecotoxicity of chemical agents” (IBAMA 1988) and used in studies for the registration of chemicals in regulatory Brazilian agencies, as well as by international protocols (Organisation for Economic Co-operation and Development. 2004) (OECD 2004) in nanomaterial assessment (Oleszczuk et al. 2015; Gao et al. 2018).

Artemia salina is a phytoplanktonic arthropod capable to respond to varied aquatic pollutants. Although it is a representative of marine ecosystems, *Artemia* has been used together with freshwater organisms in hazard concentration predictions of nanomaterials and others compounds (Nogueira et al. 2015; Castro et al. 2018).

Based on a body growth test for 96 hours, Castro et al. (2018) determined the EC50-96h of graphene oxide for *D. magna*. Among ten organisms evaluated for different trophic levels, the daphnids showed the highest sensibility to the nanomaterial with EC50-96h values of 0.58 (0.35–2.07) and 0.42 (0.076–1.09) mg/L in the absence and in the presence of humic acid, respectively.

Adam et al. (2015) indicate that the toxicity of copper oxide nanoparticles to *D. magna* is caused by copper ions formed during dissolution of the nanoparticles in the exposure medium. The majority of the particles formed large aggregates while a small fraction dissolved. This may explain the much lower effects of copper oxide nanoparticles (EC50 reproduction = 1.04 mg Cu/l) compared to copper salts (EC50 reproduction = 0.022 mg Cu/l). Although the authors found much higher metal concentration when exposed to the nanoparticles than when exposed to the salt, the Cu did not internalize in the tissues or cells to cause additional toxic effects after adsorbed on the carapace or ingested by the organisms.

Becaro et al. (2015) investigated the toxicity of silver nanoparticles stabilized with polyvinyl alcohol (PVA) toward *artemia* and *daphnia*, the results of which are displayed in Table 7.3. The advantage of application of this system is that such particles, when coated by layers of polymers, can increase the electrostatic repulsion and increase stability in suspensions.

Table 7.3 Acute toxicity of silver nanoparticles to two bioindicator microcrustaceans

Invertebrate	EC50-48h ($\mu\text{g L}^{-1}$)	95% confidence interval
<i>Daphnia similis</i>	0.26	0.18–0.403
<i>Artemia salina</i>	55.0	22.0–11.0

Adapted from Becaro et al. (2015)

The low EC₅₀-48 h values are coherent with findings in the literature and can be categorized as “very highly toxic” to the test organisms. For example, Asghari et al. (2012) found an EC₅₀ = 2 µg L⁻¹ on *D. magna* for spherical silver nanoparticles of 16.6 nm.

The results in the study by Becaro et al. (2015) also demonstrated that the PVA-modified silver nanoparticles were impregnated (resulting in agglomerates) in *Daphnia* gut, shell, and appendices, and altered eye morphology (Fig. 7.3). Considering the acute toxicity to *D. similis*, multiplied by a factor of 100 (in order to prevent the chronic adverse effects to daphnids and to protect other species), a concentration of 2.6×10^{-6} mg L⁻¹ of PVA-silver nanoparticles was determined.

Titanium dioxide nanoparticles (nano-TiO₂) are one of the most common materials used due to their photocatalytic activity in the ultraviolet (UV) region. TiO₂ occurs in different crystal phases, namely rutile and anatase, each one presenting distinct photocatalytic properties. Anatase shows high photocatalytic activity, but anatase/rutile blend tends to be more photoactive, compared to the pure phases. In this sense, Clemente et al. (2014b) reported that minimal levels of ultraviolet light could enhance the toxicity of titanium oxide to invertebrate organisms. Results of this study regarding evaluation of the organisms’ mobility are shown in Table 7.4.

Data demonstrated that *A. salina* showed superior acute sensitivity to nano-TiO₂, compared to *D. similis*, whether or not in the presence of UV light. Under common conditions of illumination, the EC₅₀-48h values surpassed 100 mg/L for *D. similis* and *A. salina*. Therefore, nano-TiO₂ can be considered practically nontoxic to these organisms (USEPA 1985). Under the UV light exposure, for *D. similis*, the EC₅₀-48h of the anatase/rutile mixture was 12 times lower than pure anatase. In the bioassays with *Artemia*, the EC₅₀-48h the anatase/rutile mixture decreased around 70-fold when UV radiation was employed, while pure anatase decreased 120-fold.

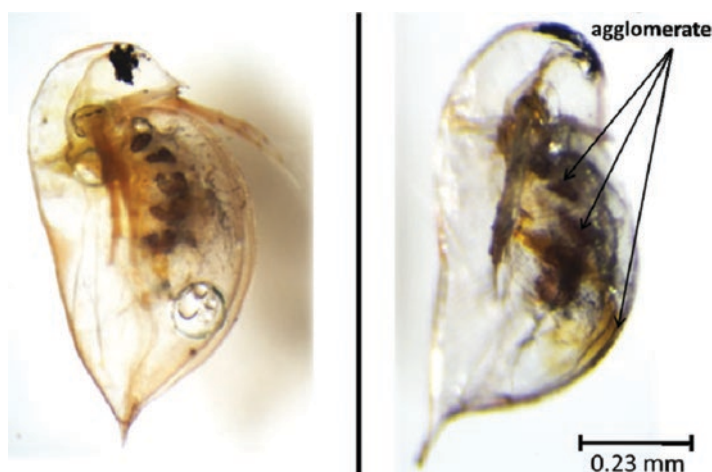


Fig. 7.3 Typical alteration observed in eye and gut of *Daphnia magna* after 24 h of silver nanoparticles exposure. (Figure adapted from Becaro et al. 2015, reprinted with permission from Elsevier)

Table 7.4 Nano-TiO₂ toxicity to two microcrustaceans under visible or ultraviolet light

		EC50-48h (mg L ⁻¹)*	
		visible light	Ultraviolet light
<i>Daphnia similis</i>	Anatase	>1000.00	750.55 (599.56–1008.92) ^a
	Anatase/rutile mixture	>1000.00	60.16 (48.30–77.94) ^b
<i>Artemia salina</i>	Anatase	480.67 (382.18–604.24) ^c	4.05 (2.35–5.62) ^d
	Anatase/rutile mixture	284.81 (213.01–374.83) ^c	4.03 (2.98–5.40) ^d

Adapted from Clemente et al. (2014a)

*The EC50-48h values are given, together with the corresponding 95% confidence intervals (in parenthesis). Different lower case letters indicate statistically significant differences between the EC50 values for the same organism

^{a,b,c,d,e} Different letters indicate statistically significant ($p < 0.05$) differences between the groups. The 96-h CE50 values for different test groups are compared with respect to control at $p < 0.05$. Each letter indicates significance with respect to control at $p < 0.05$

The results presented show that the knowledge on the nanomaterials toxic effects on phytoplanktonic and zooplanktonic offers subsidies for the establishment of public policies with regard to the use and delivery of such materials.

7.3.2 Nanotoxicity Investigation in Fishes

The adsorption of nanoparticles to fishes may occur through the gill surface, which involves similar processes to other substances. After nanoparticle uptake, target organs may include liver, spleen kidney, brain, etc., with toxic effects involving oxidative stress, ionoregulatory disturbances, and organ pathologies (Handy et al. 2008). After absorption, nanoparticle internalization occurs via endocytosis. Their toxic effects essentially depend on several factors such as the formation of aggregations, route of exposure, dose–response, exposure time, the response of the receptor organisms, and the interactions in the mechanisms involved in the physiological process of uptake (Pecoraro et al. 2018). Nanomaterial generally induces only mild acute toxicity to most adult fish, but the sensitivity may be higher for certain species and also depends on life stages. In adult animals, aquatic nanomaterial can cause respiratory and digestive epithelia irritation and causes oxidative stress. Additionally, interactions between nanomaterial (or dissolution products) and proteins can induce regulatory stress and/or developmental toxicity (Callaghan and MacCormack 2017).

Considering these aspects, it is known that the toxicity mechanisms should be studied for environmental parameters; however, there are not enough standardized sampling and methods to measure environmental influence when exposed to nanomaterial. Nanoecotoxicology has increased the availability of published data, but these are still scarce and inadequate for regulatory decision-making (Hjorth et al. 2017). Moreover, measuring and modeling of nanomaterials depend on more physicochemical parameters than conventional chemicals. Thus, standardization of dispersion methods and knowledge of dissolution kinetics are needed for interpretation and comparability of data (Tantra et al. 2011; Baun et al. 2017; OECD 2017).

Furthermore, there are no standards established for permissible levels of nano-materials in the environment (Hund-Rinke et al. 2016). Among the problems with the standard test guidelines was the difficulty in the maintenance of test solutions and exposure concentration within $\pm 20\%$ of nominal concentrations as recommended by OECD. OECD guidance suggests that variation in substance concentration larger than $\pm 20\%$ should be addressed using modified experimental procedures as a little change in pH or monitoring strategies. To overcome this, some adaptations in protocols were proposed (Petersen et al. 2015).

In some cases, however, modifying the medium may cause changes in the obtained result. Thus, for example, graphene oxide fate can be affected by pH and divalent cations as Ca^{+2} , which presence in the medium promote less stability (Wu et al. 2013; Lanphere et al. 2014; Chowdhury et al. 2015). Besides, release of silver ions was shown to be pH dependent: in zebrafish embryo medium, silver toxicity was decreased owing to the formation of silver chloro-complexes and silver nano-colloid toxicity was higher at pH 4.0 than at 7.0 or 9.0 (Shaw et al. 2016; Kennedy et al. 2017; Kataoka et al. 2018).

The fate and behavior of metallic nanoparticles in aquatic waters are complex with varied levels of variability and uncertainty. Silver may accumulate in the food chain, and the widespread use of silver nanoparticle in several applications exposes organisms (Yu et al. 2013) and raises some health concerns related to the potential risks to humans and to the environment (Zhang et al. 2013), while some studies have observed adverse effects at different levels of biological integration as reactive oxygen species (ROS) generation (Zhang et al. 2013; Gaillet and Rouanet 2015; McGillicuddy et al. 2017; Ale et al. 2018; Strużyński et al. 2014). Ellis et al. (2018) observed that silver nanoparticle stability was influenced by the seasonal variations in natural water chemistry by using a diffusion–sedimentation model to calculate silver nanoparticle migration behavior in microcosm experiments. Then, transformation, fate, bioavailability, morphology, and toxicity of silver nanoparticle are critical factors and should be considered for its environmental risk assessment (Zhang et al. 2018).

In addition, the role of intact nanoparticles along with dissolved metals is required, when toxicity cannot always be explained solely by soluble metal ions (Garcia-Reyero et al. 2014; Abramenko et al. 2018). In this way, Schiavo et al. (2018) reported that zinc oxide nanoparticle toxicity was related to Zn ions and to interactions of particle/aggregates with target organisms.

Titanium dioxide nanoparticles are widely used photocatalytic materials that show stability in water, photo and chemical stability over a wide range of pH, activation by sunlight (Woan et al. 2009, Fekete-Kertész et al. 2017). The ecotoxicology of titanium dioxide nanoparticles has been extensively studied, but not all the studies considered its photocatalytic properties, which can enhance toxicity to aquatic biota.

Clemente et al. (2013) evaluated the effects on fish (*Piaractus mesopotamicus*) exposed to different titanium dioxide nanoparticle concentrations and illumination, under visible and ultraviolet (UV) light (22.47 J/cm²/h). Titanium dioxide nanoparticles caused no mortality under any of the conditions tested, but stimulated sub-lethal effects that were induced by illumination condition. Also, fish prolonged exposures (21 days) to two different titanium dioxide nanoparticles crystal phases

(anatase and a mixture of anatase 80% and rutile 20%) were evaluated at the same light conditions. The occurrence of sublethal effects was influenced not only by illumination condition but also by titanium dioxide nanoparticle crystal phase. Pure anatase produced more oxidative damage without co-exposure to UV, while the mixture anatase: rutile caused more sublethal effects under UV (Clemente et al. 2015). Light conditions also play an important role in the dissolution processes of nanoparticles as silver nanoparticles and zinc oxide nanoparticles (Odzak et al. 2017).

Given the possibility of long-term exposure of nanomaterials, it becomes important to apply a set of bioassays in the evaluation of the potential hazard taking into account these factors. For this purpose, bioassays as survival rates, malformation, size, hatching, and biochemical biomarkers may be performed in different organisms exposed in different experimental conditions (Bour et al. 2015).

Zebrafish (*Danio rerio*) embryos are one of the most common in vivo model systems for high-throughput toxicity screening of chemicals because of their small size, rapid development, and high fecundity (Felix et al. 2016). Therefore, it is considered an excellent model for ecotoxicological (Pecoraro et al. 2018) molecular studies, embryonic development, and developmental biology (Brohi et al. 2017). Zebrafish embryo toxicity test has been shown to have results that correlate well with those of adult fish acute toxicity tests (Lammer et al. 2009; Belanger et al. 2013; Scholz et al. 2013; Busquet et al. 2014). Moreover, zebrafish embryo assays are pain-free in vivo tests, and embryonic development is perceptive to environment stress (Mu et al. 2016). In relation to titanium dioxide nanoparticle effects on zebrafish, the exposure to titanium dioxide nanoparticles anatase (TA) or an anatase/rutile mixture (TM) under UV irradiation accelerated hatching of the larvae and may have altered the equilibrium of the larvae and caused some oxidative stress. Under UV irradiation, greater mortality of the larvae of the groups exposed to TM was observed compared to TA (Clemente et al. 2014b).

Factors such as pH, ionic strength, and sunlight can interfere in the degree of toxicity and effects resulting from a combination of them are dynamic and complex. Another environmental factor that can alter nanoparticle toxicity is natural organic matter (NOM) presence. The stabilization of nanoparticles in aquatic systems due to NOM may be of concern due to their mobility. Different aquatic sources of NOM can result in variance of toxicity, and different concentrations of humic acid (HA) can affect aggregation and toxicity (Ong et al. 2017).

Recently, Clemente et al. (2017) showed that the presence of NOM changed graphene oxide toxic effects on aquatic organisms. They evaluated the toxicological effects of graphene oxide through tests with *Danio rerio* embryos, considering the washing treatment and the interaction with organic matter. Although the embryo exposure showed no acute toxicity or malformation, the larvae exposed to graphene oxide presented a reduction in the length and acetylcholinesterase activity. The authors observed that although there is a critical influence of oxidative debris (OD) on the graphene oxide material biological reactivity and hydroxyapatite interaction, the findings indicate a mitigation of material toxicity after OD removal.

Nanohybrid materials are emerging nanosystems where the properties that command the toxicity may be different compared to the isolated materials. Therefore,

nanohybrids materials have been produced for environmental technologies, making their environmental health and safety evaluation very important (Saleh et al. 2015). Recent findings indicate that the combination of titanium dioxide nanoparticles with carbon nanotubes (MWCNT) allows a greater photosensitivity (Ling et al. 2016). In order to understand the role of titanium dioxide–MWCNT in the environment, Silva et al. (2017a, b) evaluated the exposure of *Danio rerio* embryos. There was no acute toxicity, nor sublethal effects in *Danio rerio* embryos, until 100 mg L^{-1} when hatching rate and growth were observed. So, this nanohybrid material probably presents low toxicity (Côa et al. 2017). On the other hand, activated carbon from pyrolysed sugarcane bagasse (ACPB) with silver nanoparticles (ACPB-silver nanoparticles) presented environmental risks, with toxic effect to the aquatic organism *Hydra attenuata* ($\text{LC}_{50} 1.94 \text{ mg L}^{-1}$), which raises concern about the environmental implications of activated carbon materials modified with silver nanoparticles (Gonçalves et al. 2016).

7.3.3 Nanostructure Risk Assessments and Safety Analysis

An essential step in the development of products based on nanotechnology is the assessment of their potential risks and safety, including an evaluation of the potential impact of nanoparticles and practices related to their application on human or animal health and environmental destination.

While the scientific community points to a number of environmental advantages for the use of nanotechnology, on the other hand, there is still great difficulty in specifying what happens in air, water, and soil when nano-sized particles are potentially polluting waste. The replacement of some practices for these technologies and their dissemination allows one to question what are the environmental and social impacts of these technologies to the adopters and direct and indirect users. However, research in these yet incipient fields prevents us from scaling the impacts of these products to the environment and to society.

With regard to hazard analysis, as new nanomaterials are being developed at high rates and for different applications, interest has surfaced in elucidating the safety of these materials. Considering that better understanding and refinement in the formulation stage of the risk assessment problem may help reduce risk, the development of nanoparticle risk indicators can be an ally in the decision process, supporting the safety analysis of the development of nanomaterials to their release for human consumption or application to the environment.

The gap between risk assessors and researchers working in the field of nanotechnology makes difficult to develop proper indicators and reliable data to support safety regulations. The risk refers to the likelihood of an injury occurring under the conditions of use and may be reduced or increased according to the potential exposure. Therefore, the risk is the product of the hazard (adverse effect) relationship of a compound and its exposure. Consequently, a better understanding and refinement

in the formulation phase of the problem of risk assessment and detailing the potential adverse effect may help reducing the risk uncertainties for the nanoparticles.

A material at the nanometer scale has a greater relative surface area and can display quantum effects (Khan et al. 2017). As a consequence, nanostructures can present diverse characteristics, such as changes in solubility, electrical conductivity, elasticity, chemical reactivity, and bioavailability, compared to the material at the micro- or macroscale. Thus, risk and toxicological aspects are considered essential in the safety analysis processes of new nanomaterial particles, once they can be used in the food chain in applications spanning from encapsulation systems for delivery of food ingredients and those adapted for use in food packaging. In the case of food, the indicators that should draw the attention of the evaluators are related to the potential risks of altering the nanoparticle bioavailability of the food in the organism and migration of the nanoparticle present in the packages (films, coatings, among others) to food, and ultimately to the ecosystems.

Agricultural and veterinary applications focus attention on the monitoring of environmental indicators such as those related to generation of chemical residues or heavy metals in laboratories or industries of nanostructures and change in water quality in the surroundings of the industries that produce nanostructured products, for example (Rajaganapathy et al. 2011; Alves et al. 2016; Ali et al. 2019). In the environmental case, the indicators that should draw the attention of the evaluators are related to the potential risks associated with persistence and bioaccumulation of the nanoparticle in the environment, soil, and water contamination due to the dispersion of nanoparticle applied in agriculture. In addition to the possibility of a nanoparticle presenting toxicity and/or ecotoxicity, special attention for inappropriate disposal of products or wastes containing nanoparticles should be given. These indicator tolls tend to reduce the time lag between the production of knowledge or technology and the development of criteria for the safety evaluation for the environment and society, focusing its effective use by the public power and the productive sector, contributing to safety development and use of nanotechnology.

7.4 Conclusion and Final Remarks

In this chapter, we presented some results regarding the syntheses and ecotoxicity of several important nanostructures (including silver nanoparticles, titanium dioxide nanoparticles, graphene oxide, etc.), which are nowadays widely applied in technological applications. Focus was given on toxicological aspects of these nanostructures toward algae, microcrustaceans, and fishes, which represent three trophic levels including one primary producer (algae) and primary (microcrustaceans) and secondary consumers (fish). Results revealed that the nature, shape, and medium where the nanostructure reside could strongly influence their toxicities toward distinct aquatic microorganisms.

Regarding ecotoxicity, it is important to study the effects of sedimentation in real situation, which sometimes are not taken into account during laboratory tests.

Repeated exposure studies during multiple years are important to uncover and avoid nanostructure accumulation in environmental sediments over time (Bundschuh et al. 2018), which can affect species at various trophic levels (Bour et al. 2016; Bhuvaneshwari et al. 2017) and cause deleterious effects to human health and nutrition (Gardea-Torresdey et al. 2014). For instance, according to Kim et al. (2016), titanium dioxide nanoparticles show superior movement in the sediment than in the water and can be retained through aquatic food chains after a consecutive low-dose exposure than after a single high-dose exposure. Therefore, an increase of models used to assess the fate, transport, and effects of nanostructures in aquatic systems is highly demanded. Additionally, there are some barriers to the effective action of risk assessment and management, including lack of nano-specific regulations and validated and accessible methods for safety testing, reliable information on commercial use, etc. (Miller and Wickson 2015; Hu et al. 2016; Mouchet et al. 2016). Moreover, substantial methodological limitations must be overcome in order to allow better quantification of nanostructure and biological uptakes in aquatic environments.

Acknowledgments The authors thank the financial support from FAPESP (2017/12174-4), CNPq, MCTI-SisNano, FINEP, Embrapa, and Nanotechnology Network for Research in Agriculture (Rede AgroNano).

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Chapter 8

In Vitro Methodologies for Toxicological Assessment of Drug Delivery Nanocarriers



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Abstract Drug delivery nanocarriers (DDNCs) are very suitable systems in drug transport to site-specific targets. The physicochemical characteristics that make DDNCs promising systems for disease therapy can be also correlated with potential adverse effects. In the development of functional DDNCs, both efficacy and safety assessment are crucial. Until now, there is no established testing strategy to perform nanomaterial toxicological evaluation. Several *in vitro* cell culture models and testing protocols are commonly applied for the safety profile assessment of a nanocarrier; however, a large amount of disperse and conflicting data are generally provided in scientific literature. Thus, systematic research on the toxicological effects of nano-based systems is crucial due to their increasing development, production, and usage. This chapter highlights the critical aspects regarding nanotoxicity assessment, addressing the most important analytical strategies/techniques and endpoints, from the physicochemical properties' characterization of DDNCs to their biological influence and behavior in cells and organisms.

Keywords Nanotoxicity · Nanocarriers · *In vitro* assays · Nanocarrier characterization · Analytical methods

8.1 Introduction

Nanotechnology deals with the manipulation of matter, in atomic and molecular levels, at nanoscale leading to alterations in its properties (Zarbin 2014). During the last decades, the achieved breakthroughs in nanotechnology have also been translated in new materials, developing more effective tools for therapy and diagnostics with applications in the biomedical field (Cole and Holland 2015). Nanotechnology medical applications, also referred as nanomedicine, intends to address the diagnostic, treatment and prevention of acute and chronic diseases, leading to the development of drug delivery nanocarriers (DDNCs). DDNCs engineering and development main goals consist of (i) solubility and bioavailability improvement of hydrophobic drugs; (ii) drugs circulatory time increase, avoiding metabolic processes before reaching therapeutic site; (iii) diminishing side effects with the decrement of administered doses; (iv) drug-targeting to specific tissues and cells or individual pathogens and biomolecules; and (v) controlled drug release (Moghimi et al. 2005). To date, there are already several established and commercially available nanotechnology-based products and formulations. Biodegradable “soft” platforms, such as liposomes, micelles, emulsions, and/or other polymeric and protein nanostructures, are preferred for therapeutic delivery applications. Examples of FDA-approved are Abraxane®, Doxil®, DaunoXome®, and Copaxone® (Dong et al. 2016).

The growing development, production, and use of engineered nanomaterials (NMs) is inevitably leading to direct and indirect effects in humans, as well as emissions into the environment. Therefore, the assessment and evaluation of the potential risks for human health and environment as a result of exposure to NMs is also a matter of concern. The term nanotoxicology was coined for the first time in 2004 by

Donaldson et al. (2004) to establish a new subarea in the toxicology field with focus on the knowledge of NMs-induced toxicity. Thus, nanotoxicology is defined as the “study of adverse effects of NMs on living organisms and ecosystems, including the prevention and amelioration of such adverse effects” (Cattaneo et al. 2010).

Hazard characterization and toxicity testing of NMs are carried out using in vitro and in vivo assays, for the establishment of dose–response relationships, also considering different degrees of exposure. NMs toxicological characterization must be considered on the primary stages of new material development with the purpose to predict the biological response obtained with the manipulation of several parameters. The unique properties of NMs and their higher reactivity, which differ from conventional sized materials, may reflect on toxicity (Patel and Shah 2017). In fact, several physicochemical parameters, such as chemical composition, size and surface area, surface functionalization and charge, shape, and dissolution profiles (Seitz et al. 2014; Mendes et al. 2014; Huang et al. 2011; Gonzalez et al. 2014), are responsible for biological effects, thus a casuistic approach for studying their impact on human health is required.

NMs interact with biological systems in several manners leading to adverse effects, which are summarized in Table 8.1. Thus, a large number of toxicological endpoints must be considered for hazard characterization. However, traditional

Table 8.1 Potential nanomaterials/nanomedicines induced adverse effects

Nanomaterial/nanomedicine effects	Pathophysiological outcomes
Oxidative stress	Protein, DNA and lipid damage, phase II enzyme induction, inflammation, mitochondrial alterations
Protein denaturation, degradation, and aggregation	Loss of enzyme activity, autoantigenicity, aggregates
Nuclear uptake	DNA damage, nucleoprotein clumping, autoantigenicity
DNA damage	Genotoxicity and carcinogenesis
Mitochondrial dysfunction	Inner membrane damage, permeability transition pore opening, energy failure, apoptosis, necrosis, cytotoxicity
Inflammation	Inner membrane damage, permeability transition pore opening, energy failure, apoptosis, necrosis, cytotoxicity
Endothelial dysfunction, effects on blood clotting	Atherogenesis, thrombosis, stroke, myocardial infarction
Reticuloendothelial system uptake	Sequestration and storage in liver, spleen, lymph nodes, organ enlargement, and dysfunction
Neuronal tissue uptake	Central and peripheral nervous systems damage
Phagocytic function perturbation, particle overload, mediator release	Chronic inflammation, fibrosis, granulomas, interferences with infectious agent clearance systems
Neoantigens generation, breakdown in immune tolerance	Autoimmunity, adjuvant effects
Altered cell cycle	Proliferation, cell cycle arrest, senescence

Adapted from Ciappellano et al. (2016)

methodologies are often not suitable for nanotoxicology assessment due to the possible interferences that occur while common toxicological assays are preformed (Shatkin and Ong 2016). Therefore, specific adaptations to these assays or alternative ones should be also considered to overcome the limitations of usual and established testing.

In this chapter, we intent to highlight the most relevant aspects to be considered in nanotoxicological studies, taking as an example DDNCs. Starting with the reference to the main physicochemical parameters that are primarily evaluated, we will review the different analytical methodologies available for the assessment of NMs induced toxicity, from the classical tests and evaluation protocols to novel “omics” techniques, as well as to discuss the challenges to overcome in the future in this matter.

8.2 Drug Delivery Nanocarriers (DDNCs)

Established drug formulations often exhibit limitations related to pharmacokinetics and barrier transport, such as poor solubility, permeability, and bioavailability (Szabo and Zelko 2015). The identified limitations of classical drug therapy have been mentioned in Fig. 8.1 (Soliman 2017).

The attempt to overcome these problems was one of the most relevant challenge of pharmaceutical industry in the last years. Apart from this, it was also the reason of the investigation for nanoparticles based on drug delivery platforms, which can be a suitable vehicle to overcome the identified limitations (Blanco et al. 2015). DDNCs have emerged offering new possibilities and profiles for drugs which have not been explored at their maximum potentiality and/or to re-direct their use in different administration routes.

Engineering of DDNCs with proper physicochemical properties will allow the combination of different NMs with a high biodegradability and biocompatibility (Bilia et al. 2014). The main properties of DDNCs have been summarized in Fig. 8.2. To achieve a therapeutic effect, they must overcome biological barriers (Bilia et al. 2014).

Besides targeted drug delivery, imaging and diagnosis are also applications influenced by the surface properties of the carriers and have been exploited for (i) early stage cancer diagnosis (Liu et al. 2007), (ii) assessment of real-time treatment (Rowland et al. 2012), (iii) high-concentration site-specific drug delivery (Lee and Wong 2011); (iv) mutations detection (Youns et al. 2011), (v) identification of new targets for clinical research (Heidel and Davis 2011).

Different types of DDNCs have been developed depending on the type of NMs, screened and selected according to the properties of the drug to be loaded (Fig. 8.3).

Different DDNCs, with stability related to their functionality, are classified and characterized according to the chemical nature of the drug carrier. As shown in Fig. 8.3, polymeric nanoparticles are reservoir-based nanosystems composed of an inner liquid phase surrounded by a polymeric layer that can control the release,

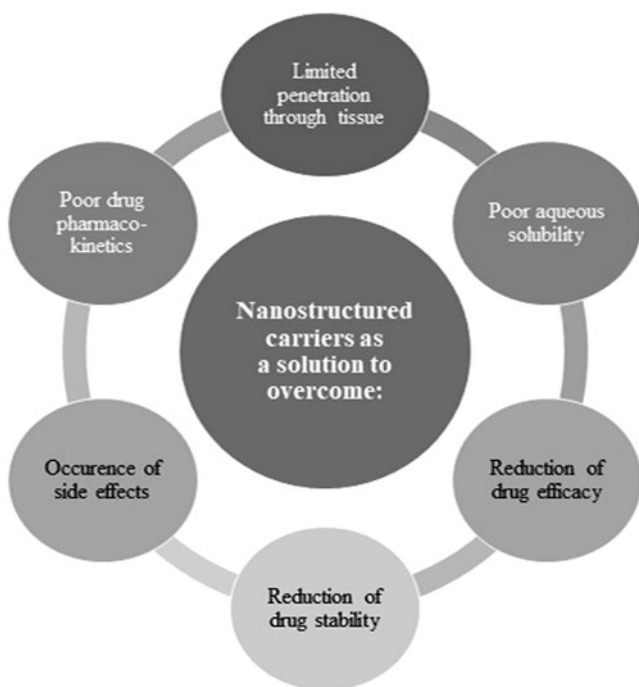


Fig. 8.1 Identified limitations of classical drug therapy expected to be overcome by using nanostructured carriers

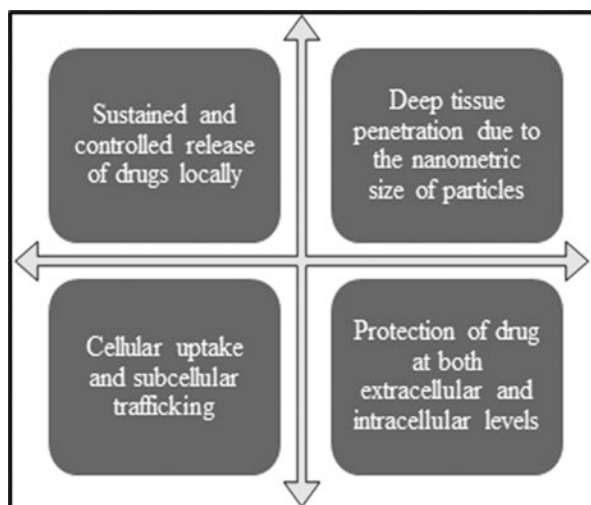


Fig. 8.2 Main properties of nanostructured carriers to overcome biological barriers for targeted drug delivery

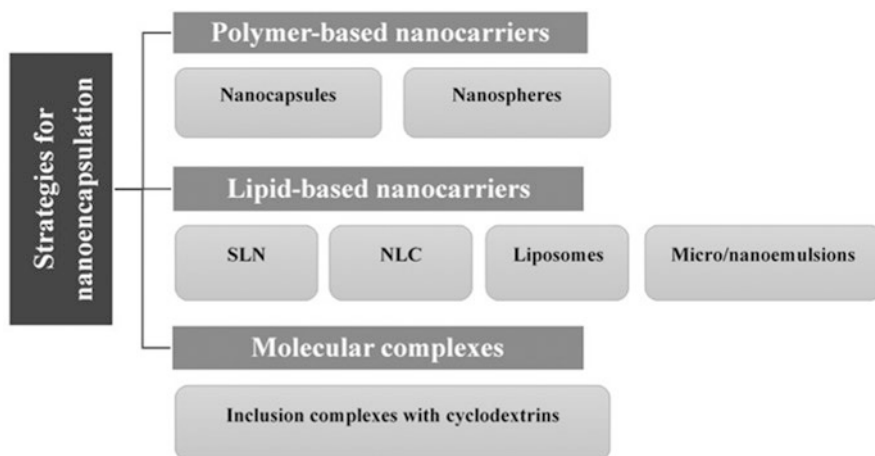


Fig. 8.3 Examples of nanostructured carriers based on the type of nanomaterial. (Adapted from Bilia et al. 2014)

whereas nanocapsules and nanospheres are matrix-based nanosystems, from which the drug is released mainly by diffusion.

The use of DDNCs offers several advantages. As therapeutic materials, they provide great improvements in solubility increase, dissolution rate enhancement, and drug bioavailability improvement (Gaba et al. 2015). These advantages have been already proven and useful in distinct therapeutic areas, namely some of those consider critical in public health, namely, antibiotic resistance and chemotherapy (Jaiswal et al. 2016).

Nanoparticles have also the ability to pass through biological barriers, namely, the blood–brain barrier (BBB) or the blood-retinal barrier (BRB), gaining interest for therapeutical approaches in specific diseases, such as brain and retinal diseases. This particular characteristic allows an increase in the residence time of therapeutic drugs in target organs leading to better therapeutics' results. Moreover, the physical and chemical properties of DDNCs can also enhance the bioavailability of therapeutic agents in local and systemic administration (Jo et al. 2015).

Considering the development of these nanosystems, pharmaceutical technology may achieve the possibility for a better controlled therapeutic agent delivery and targeting to the organs or tissues (Rowland et al. 2012). Thus resulting in (i) drug stability increase and toxicity decrease, resulting in a biochemical protection in the human body (Mishra et al. 2013); (ii) the reduction of adverse effects—once the interactions of these with the biological surroundings are less probable (Rowland et al. 2012); (iii) long circulation times with an increase of the drug lifetime (Mishra et al. 2013); (iv) biological degradation of the nanoparticles (Mishra et al. 2013); (v) more effective production using simpler and less expensive methods; (vi) an increase in storage lifetime related to the improvement of the compounds stability (Duncan and Gaspar 2011).

All the advantages aforementioned and related to DDNCs as therapeutic agents can provide an outstanding solution for drug efficacy and safety improvement, as well as a great technological advance in the biomedical field (Demetzos and Pippa 2014).

8.3 Nanomaterials Physicochemical Parameters Evaluation

Toxicological research and assessment for engineered DDNCs must consider the highly dynamic physicochemical properties of NMs. NMs synthesis is a process that results in several chemical and physical transformations and/or modifications, which will also influence the interaction mechanisms with biological systems and their resulting toxicity. Nowadays, new NMs are being developed with large chemical and structural diversity, which leads to highly variable interactions with living systems and, subsequently, to difficulties in the establishment of toxicity evaluation standard protocols (Hassan and Singh 2014). However, there is a commonly agreed minimum set of NMs physicochemical properties and their characterization studies for nanotoxicity assessment. These properties include chemical composition, size, shape, surface characterization, crystallinity, and agglomeration/aggregation state (Kim et al. 2014).

The first critical physicochemical property that influences NMs toxicity is their chemical composition, once single elements chemical reactivity and toxicity in biological media differ when they are arranged in a nanosystem. Common analytical technologies that are suitable for NMs chemical composition include spectroscopy analysis (e.g., X-ray photoelectron spectroscopy (XPS), Raman spectroscopy, energy dispersive X-ray analysis (EDX), inductively coupled plasma (ICP), or Fourier transform infrared spectroscopy (FTIR)) and nuclear magnetic resonance (NMR) (Kim et al. 2014).

The size of the nanostructured carriers is one of the features, which differentiates them completely from a range of conventional drug carriers. The compounds considered at a nanoscale are featured in the colloidal size range, between 0.1 nm and 500 nm/1 μm (Souto et al. 2007, 2020a; Mahant et al. 2020); however, for systemic administration in therapeutics, nanoparticle size that is considered suitable ranges between 2 and 200 nm (Jo et al. 2015). Size and shape are crucial factors that define both preferential internalization mechanisms and uptake efficiency, conditioning thus the toxicological behavior of NMs in biological environments. Thus, the size determination is an essential measurement after production of nanocarriers, and a variety of appropriate analytical techniques includes microscopy techniques (transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM)) and X-ray diffraction (XRD) (Kim et al. 2014, 2015). However, the most commonly used technique is dynamic light scattering (DLS), which allows the detection of the particle size (Z-Ave) and the polydispersity index (PDI), at the same time. These parameters are essential during the optimization

process and the assessment of stability of the nanodispersions, which should reach a high monodispersity (Fangueiro et al. 2013).

The surface properties of NMs (i.e., surface area, chemistry and charge) will also have a strong influence on their interaction with cellular membranes, which is considered a first step for induced cytotoxicity (Jo et al. 2015). Regarding surface chemistry, NMs engineering often involves the performance of chemical modifications for surface functionalization. These modifications might have serious effects on their toxicological profile once while some functional groups can offer greater biocompatibility to NMs, on the other hand, their incorporation on the surface can lead to greater toxic potential. Analytical techniques for NMs surface chemistry determination are mainly the same, which were described for chemical composition analysis (Surassmo et al. 2015). In terms of surface charge, zeta potential is the property that is accessed, and it is crucial to always determine whether NMs are positively or negatively charged. For instance, several reports have shown that positively charged NMs induced more toxic effects on cells than their negatively charged counterparts (Bhattacharjee et al. 2013). Zeta potential is usually determined by light-scattering electrophoresis or electro-acoustophoresis methodologies. Finally, surface area and porosity can be determined by the Brunauer–Emmett–Teller analysis method, based on gas adsorption/desorption isotherms (Shin et al. 2015).

The crystallinity of NMs might also be a property with a relevant role in their physicochemical and toxicological behaviors. Variations in chemical stability of NMs may lead to different biological response and are correlated to the differences in the orientation of the atoms. Information about the crystallographic structure of NMs are mainly collected by XRD, which is the most common technique used to define crystals pattern, position, intensity, and shape of the diffraction peaks (Kim et al. 2014).

8.4 In Vitro Toxicological Assessment of Nanomaterials

Considering the previously commented challenging factors associated with the interaction of NMs with biological systems, two different approaches may be applicable for nanotoxicity assessment, namely, (i) the commercially available kits that make use of conventional in vitro protocols to evaluate a specific cellular endpoint likely to be modified by the NMs under testing, and (ii) advanced analytical methods that assess specific toxicological effects of NMs. Gunsolus and Haynes (Gunsolus and Haynes 2016) reviewed the most used analytical techniques in NM toxicity research, taking into account the physicochemical properties of NMs and their potential biological impact.

The use of cell cultures for the recording of toxicological endpoints evaluates any physiological and/or biochemical changes induced by NMs (e.g., oxidative stress promotion, inflammation induction), as well as the risk of cyto-/genotoxicity (Love et al. 2012; Doktorovova et al. 2014a). Examples of the most common in vitro

Table 8.2 Most common in vitro assays to assess nanotoxicity

Endpoint	Cellular process	Assays
Cell viability	Metabolic activity	MTT, MTS, XTT, WST-1, Alamar Blue
	ATP content	ATP assay
Cell permeability/ tight junctions functionality	Membrane integrity	TEER
	Apparent permeability	TEER, Lucifer Yellow, Mannitol, Dextran, Target Molecule titration by means of HPLC
Cell death	Apoptosis	Caspase-3/7 activation, Annexin V, FACS, ELISA and Immunoblotting
	Necrosis	LDH, trypan blue, neutral red, propidium iodide by FACS, ELISA, and immunoblotting
	Autophagy	Electron microscopy, optical microscopy, immunoblotting, immunoprecipitation, and immunofluorescence
Oxidative stress	Reactive oxygen species (ROS) generation	ROS assay (e.g., H2DCF-DA), FACS
	Lipid peroxidation	TBA assay for malondialdehyde, HPLC, spectrofluorimetry, gas chromatography–mass spectrometry and F2 isoprostanes
	Protein carbonylation	DNPH assay, immunoblotting, and mass spectrometry
	Glutathion (GSH) depletion	GSH/GSSG ratio
	Superoxide dismutase activity and expression	Nitro blue tetrazolium and immunoblotting and PCR
Genotoxicity	DNA damage	Comet assay, micronuclei presence, TUNEL assay
Immunogenicity	Alteration of immune system	Enzyme-linked immunosorbent assay or real-time PCR, FACS, microarray, CFU-GM and CFU-E, whole blood cultures, hemolysis test, thrombogenicity assay (activated partial thromboplastic time assay, thrombin generation assay, blood clotting time assay, calibrated thrombin generation assay), phagocytosis assay, DC maturation

Adapted from Ciappellano et al. (2016)

assays performed are listed in Table 8.2, and they can be used for assessing nanocarrier-induced toxicity on both differentiated and undifferentiated cells.

In this section, we review a series of well-established assays for in vitro assessment of NMs toxicity based on the previous classification. We focus on the determined protocols, describing the results expected, as well as their main limitations.

8.4.1 Cytotoxicity and Cell Viability Assays

Cytotoxicity and cell viability assays are the most common studies to assess the toxicological profile for a given compound or xenobiotic. These are important in the definition of dose–response relationship and identification of sub-lethal concentrations for mechanistic studies. Definition of dose–response curves is fundamental for extrapolating relevant toxicological parameters, such as minimum effective concentration (MEC), half-maximal effective concentration (EC₅₀), and maximum effect ($E_{\max}$). These parameters can be used to compare toxicological profiles of different formulations, as well as to investigate the contribution of constitutive components in modulating nano-based formulation toxicity (Gunsolus and Haynes 2016).

Cytotoxicity assays measure cell death after a treatment with a cytotoxic drug or compound, and they are generally related with cell death mechanisms such as necrosis and apoptosis (i.e., programmed and accidental cell death). These mechanisms reflect the ability of a drug molecule to trigger intracellular suicide mechanisms or destroy cells. Not very different than any other compounds or materials, NM-induced cell death assessment focuses on examining the mitochondrial membrane integrity or potential by the assessment of the apoptotic protein levels or DNA fragmentation (Akhtar et al. 2014). While apoptotic proteins activation are apoptosis indicators, cell membrane damage is usually considered a necrosis marker (Love et al. 2012).

Membrane damage can be determined by measuring either the release of cytosolic molecules as lactate dehydrogenase (LDH) enzyme or the entrance or uptake of exterior molecules that can function as probes, such as trypan blue, propidium iodide, or neutral red dye. The LDH assay measures the activity of the enzyme released in cell culture medium after contact with the tested NM (Jing et al. 2015). The released LDH converts pyruvate into lactate with the consequent reduction of NADH into NAD⁺. Therefore, a decrease of NADH absorption peak in extracellular medium corresponds to an increase of extracellular LDH concentration (Caballero-Diaz and Cases 2016). Extracellular LDH content can usually be quantified by colorimetric methods based on the conversion of tetrazolium salt into soluble dyed formazan, which are also available in commercial kits (Love et al. 2012). As previously mentioned, trypan blue and propidium iodide are charged dyes able to enter damaged membranes and then excluded from viable cells, thus presenting selectivity allowing them to be functional probes (Love et al. 2012). Trypan blue dye is suitable for conventional spectrophotometric techniques with an absorption peak at 605 nm, and it can generally serve for manual counting of alive/dead cells (Monteiro-Riviere et al. 2009). On the other hand, propidium iodide is a fluorescent dye that enters cells with disrupted membrane and intercalates into DNA and double-stranded RNA. Suitable for a wide array of techniques, such as fluorescence microscopy, confocal laser scanning microscopy, flow cytometry, or fluorimetry, upon binding to nucleic acid molecules, propidium iodide fluorescence increases 20- to 30-fold (Chueh et al. 2014; Jorgensen et al. 2017). Neutral red is an uncharged dye under physiological conditions and is retained into lysosomes of viable cells

following protonation by acidic environment. The neutral red uptake (NRU) assay is based on the ability of viable cells to incorporate and bind neutral red dye. Since ATP is essential for maintaining pH gradient into lysosomes, the reduced ability to retain neutral red dye indicates cytotoxicity (Hu et al. 2015).

Cellular viability is mostly dependent on cellular damage after cell treatment with NMs (Souto et al. 2020b, c; Silva et al. 2019a). In turn, cellular damage can also be correlated to a reduction in the cellular metabolic activity and proliferation (Silva et al. 2019b, c). Depending on investigated cellular process, several in vitro assays used to evaluate cellular viability after a xenobiotic exposure (Yang et al. 2016). In vitro assays such as MTT, MTS, XTT, or WST-1, based on the cellular reduction of tetrazolium salts to dyed formazan-based products by mitochondrial dehydrogenases, can give a direct indication on cell viability and indirectly on cell proliferation (Gunsolus and Haynes 2016). Differences between assays rely on chemical composition of tetrazolium salts. While MTT is a positively charged molecule that readily enters viable cells and is converted to an insoluble formazan product, MTS/XTT/WST-1 are negatively charged molecules, which are promptly converted into soluble formazan products. Thus, in MTT assay, formazan precipitates require previous solubilization before absorbance reading, preventing the possibility to combine different assays on the same well (Riss et al. 2011). On the other hand, soluble formazan products obtained using MTS/XTT/WST-1 assays allow the sequential analysis of treated cells with other in vitro assays (Riss et al. 2011). Similar to tetrazolium salt-based cell viability assays, AlamarBlue® is also a proven cell viability indicator based on the reduction of resazurin into the bright red fluorescent resorufin by viable and metabolically active cells (Love et al. 2012).

ATP content, related to the metabolic activity of viable cells, can also be used as a valid marker to estimate proliferation and cytotoxicity of cultured mammalian cells (Fisichella et al. 2012; Costa et al. 2016). Luminescence-based ATP assay is a fast, sensitive and suitable in vitro test for high-throughput screening, as the luminescent signal usually reaches a steady state within 10 min after the addition of the specific reagent. This assay requires cell lysis, hampering further testing on the same cells (Riss et al. 2011).

To validate in vitro assay results, proper controls should be considered. Controls can be classified as negative (e.g., vehicle/dispersant, excipient, or soluble components) or positive (Stone et al. 2009). Negative controls are usually represented by cell culture medium or incubation buffers such as Hank's Balanced Salt Solution (HBSS) (Monteiro-Riviere et al. 2009; Costa et al. 2016), and the most common positive controls for evaluating cell viability are acetaminophen and sodium deoxycholate (Gunsolus and Haynes 2016).

8.4.2 Oxidative Stress

Oxidative stress is an important parameter for evaluating NMs toxicity (Caballero-Diaz and Cases 2016; Czerska et al. 2015). Oxidative stress is defined as a biochemical imbalance that leads to the production of free radicals (pro-oxidants) and the molecules responsible for their removal (antioxidant defense mechanisms) (Czerska et al. 2015).

Reactive oxygen (ROS) and nitrogen (RNS) species are considered the pro-oxidant markers of oxidative stress, and for their detection several molecular probes have been developed (Gunsolus and Haynes 2016; Jing et al. 2015; Czerska et al. 2015). The non-polar 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA) is one of the mainly used probes (Aranda et al. 2013). Once internalized, DCFH-DA is converted into the polar derivative DCFH by cellular esterases and subsequently oxidized to a highly fluorescent 2',7'-dichlorofluorescein (DCF) by radical species. As both ROS and RNS are responsible for its formation (Crow 1997), DCF fluorescence is an indicator of oxidative stress without identifying any specific radical species (Marchesi et al. 1999). This molecular probe can be monitored by fluorimetry, flow cytometry, or by fluorescence microscopy, making it highly flexible for the observation of the formation of radical species (Love et al. 2012; Stone et al. 2009).

Considering also the antioxidant defense mechanisms, the monitorization of the regulation of biological oxidant molecules can also provide insight on the oxidative stress imbalance. For instance, glutathione (GSH) is an important antioxidant in plants, animals, fungi, and some bacteria and archaea, playing a role in preventing damages caused by reactive oxygen species. It exists in both reduced (GSH) and oxidized (GSSG) states, and in healthy conditions more than 90% of the total glutathione presents in its reduced form. Thus, an increased GSSG-to-GSH ratio is considered indicative of oxidative stress (Doktorovova et al. 2014b, 2016). Several *in vitro* assays based on colorimetric, fluorescent, or luminescent molecular probes can be used to estimate GSSG-to-GSH ratio (Meloni and Nicolay 2003). Moreover, the activation and upregulation of the antioxidant enzyme superoxide dismutase (SOD) represents another cellular defense against oxidative stress. SOD activation can be assessed measuring superoxide-dependent conversion of substrates such as dihydroethidium (DHE) or nitroblue tetrazolium (NTB) into the red fluorescent DHE or to blue formazan, respectively (Love et al. 2012; Stone et al. 2009). SOD upregulation can be quantified by immunoblotting techniques (Love et al. 2012).

While molecular probes such as DCFH-DA directly measure radical species formation, oxidative stress generation can be indirectly investigated by analyzing oxidative-dependent damages to biological molecules as proteins, lipids, sugars, and nucleic acids. For instance, lipid peroxidation and protein carbonylation protein oxidation reactions promoted by ROS and their products can be considered as oxidative stress biomarkers. Depending on the biomolecular reaction, specific mass spectrometry and chromatographic techniques have been adopted, as well as

advanced “omics” approaches, later discussed in Sect. 8.4.5 (Suzuki et al. 2010; Calvano et al. 2014).

8.4.3 *Proinflammatory Activity and Immunological Response*

NMs hold the capacity to interact with the immune system and induce immunological responses (Shegokar et al. 2018). To study NM-induced immunotoxicity, several in vitro assays have been developed. Some of them, as hemolysis and granulocyte macrophage colony-forming units (CFU-GM), have been already standardized by American Society for Testing and Materials International for immunotoxicity evaluation of NMs (Dobrovolskaia 2017).

However, immune response in cells is triggered by the release of cytokines, mainly expressed by macrophages, that play a key role in the regulation of the immune response, inflammatory reaction, and phagocytosis (Omar et al. 2015). Production and release of cytokines are well-known responses of immune system and represent a widely accepted way to estimate immunostimulation of substances. Depending on types and levels of cytokines, it is possible to disclose the mechanisms of immunostimulation. Whole blood, peripheral blood mononuclear cells (PBMC), and human-derived MM-6-cell line are the most common cell culture models for analyzing cytokines. In particular, whole blood cultures are widely used in the drug development industry, and they are recommended by the European Food Safety Authority Scientific Committee (Dobrovolskaia and McNeil 2013). Cytokine production and release are usually assessed by ELISA (Gunsolus and Haynes 2016). ELISA takes advantage of antibody/antigen recognition for detecting specific proinflammatory and antiinflammatory cytokines such as IL-8, IL-1 β , IL-6, and TNF- α (Ledur et al. 1995). By means of enzyme-conjugates antibodies such as horseradish peroxidase and alkaline phosphatase, it is possible to quantify released cytokines by recording fluorimetric, colorimetric, or luminescent signals (Ledur et al. 1995). The release of proinflammatory cytokines could also be assessed by monocyte-derived dendritic cell maturation assay. Indeed, dendritic cells, relevant for their antigen-presenting activity, mature following exposure to inflammatory cytokines (e.g., TNF- α) or pathogen-associated molecular patterns (PAMPs) (e.g., bacterial LPS). This assay is not only useful to screen nanocarrier interference on DC maturation process, but it could also be used to study their potential cytotoxicity (Sousa et al. 2017). Besides, dendritic cells maturation could be applied to nanoparticle-based vaccine formulation in vitro study (Knuschke et al. 2013; Tomić et al. 2014). The involvement of specific mechanisms in modulating NM-induced immunotoxicity can also be investigated by analyzing differentially expressed genes by microarrays (Love et al. 2012).

To complement cytokines analysis, hemolysis, complement activation, and thrombogenicity testing are also recommended. In vitro hemolysis tests showed a good correlation with in vivo tests, independently from blood origin species and anticoagulant (Dobrovolskaia and McNeil 2013). Complement activation is the part

of innate immune system responsible for enhancing the ability of antibodies and phagocytic cells to clear pathogens from an organism, and its *in vitro* evaluation is strongly dependent on the applied matrix (Neun and Dobrovolskaia 2011). Thrombogenicity is a complex process encompassing multiple cell types (thrombocytes, leucocytes, endothelial cells) and plasma coagulation factors; therefore, a multiple-assay approach is highly recommended (Myerson et al. 2011).

8.4.4 Genotoxicity

Genotoxicity is considered a fundamental *in vitro* endpoint in nanotoxicology, due to its correlation with cancer risk (Doktorovova et al. 2014a, c; Souto et al. 2020d). There is no single test established to detect all genotoxins, thus when performing genotoxicity assays, a multiple approach should be carried out. However, within the selection of a suitable test battery, critical endpoints should be considered, namely, gene mutation, structural and numerical chromosome aberrations (Bastús and Punes 2017), and generally, those endpoints can be assessed by the following *in vitro* assays: (i) *in vitro* mammalian cell gene mutation test; (ii) *in vitro* mammalian chromosome aberration test; and (iii) *in vitro* mammalian cell micronucleus test (Rasmussen et al. 2016). The first assay, cell gene mutation test, is usually used to detect gene mutations at hypoxanthine-guanine phosphoribosyl transferase (HPRT), and at a transgene of xanthine-guanine phosphoribosyl transferase (XPRT). The HPRT and XPRT genes mutation tests detect different spectra of genetic events. The chromosome aberration test identifies structural chromosome aberrations—in chromosome or chromatid—caused by chemicals and other xenobiotics. After treatment, stained metaphase cells are analyzed microscopically for the presence of aberrations. Lastly, the micronucleus test can detect the presence of micronuclei in cytoplasm of interphase cells originating from acentric chromosome fragments or whole chromosomes unable to migrate during cell division (Rasmussen et al. 2016; Bastús and Punes 2017). This chromosomal alteration concerns cells undergoing mitosis, and the use of cytochalasin B as cytokinesis blocker permits the analysis of those cells that have completed mitosis (Manshian et al. 2015; Branica et al. 2016).

On the other hand, as a simpler alternative DNA damage testing can also be considered for genotoxicity assessment. To evaluate DNA fragmentation, the marker of DNA damage, two different *in vitro* assays are reported: comet assay and TUNEL assay (Silva et al. 2019a). Comet assay is based on identification and quantification of comet tails after gel electrophoresis of lysed cells, and the brighter and longer the tail, the higher the level of damage is (Love et al. 2012; Stone et al. 2009; Burlinson 2012). TUNEL assay consists in a method for detecting apoptotic DNA fragmentation relying on the binding of terminal deoxynucleotidyl transferase (TdT), tagged with a fluorochrome or another marker, to 3'-hydroxyl termini of DNA double-strand breaks, thus detecting damaged cells (Love et al. 2012; Caballero-Diaz and Cases 2016).

8.4.5 “Omics” Methodologies

Some biomolecules expression, including genes, mRNA transcripts, proteins, and metabolites, can be affected in an organism after a xenobiotic exposure, providing information about the specific pathways that have been altered as a consequence of this exposure. These molecular indicators can serve as biomarkers not only to provide insights into the triggered toxicity mechanisms but also to provide information about the modulation of the general molecular response of a cell, tissue, or organism in response, for instance to NMs (Klaper et al. 2014).

Innovative and advanced technology platforms such as genomics, transcriptomics, proteomics, lipidomics, and metabolomics have been lately applied to investigate mode of action and mechanisms of nanomedicines. “Omics” technologies can also represent a valuable tool to improve the evaluation methods of NMs toxicity signatures. These approaches allow the simultaneous detection and identification of many different molecules, including their minor parts, presented, expressed, or altered in a biological system following xenobiotic exposure. Furthermore, the assessment of the behavior of these molecules can also provide information about the interactions and interferences of the xenobiotic with biochemical pathways (Azhdarzadeh et al. 2015).

Gene expression can provide a sensitive endpoint for toxicity as it suffers alterations in response to external stimuli, such as NMs. Given genomics and transcriptomics studies, genomics refers to the study of the genome function and structure, while transcriptomics is the study of transcriptome, namely, the messenger RNA molecules in biological systems using microarray technology to monitor the global changes occurring into cells after a xenobiotic exposure. For instance, genomic/transcriptomic studies on the effect of NMs on gastrointestinal tract have been carried out with whole human genome oligo microarray using human cell-based systems (Fisichella et al. 2012; Bouwmeester et al. 2011).

Gene expression alteration studies are usually complemented by the studies on the alterations induced on protein, lipid, and metabolic mechanisms and pathways. Proteomics refers to the large-scale study of proteins, focusing on their structures and functions. In vitro proteomics provides useful information aiming to identify toxicity biomarkers for oxidative stress and cell death mechanisms. To study the impact of nanoformulations on proteome, along with two-dimensional gel electrophoresis (Sturla et al. 2014), advanced mass spectrometry-based techniques, such as stable isotope label-free quantitative mass spectrometry, have been developed and applied (Calvano et al. 2014; Bouwmeester et al. 2011; Ng et al. 2015). The cellular lipid pathways and networks in biological systems are studied through lipidomics. The analysis of the complete lipid profile within cells, tissues, or organisms after a xenobiotic exposure is also an important tool in the understanding of the interactions between NMs and biological systems. Making use of different types of mass spectrometry (Paglia et al. 2015), it is possible to study alterations in lipid compositions and possible lipid modifications induced by oxidative stress (Hinterwirth et al. 2013; Vaz et al. 2015). Physiological changes within cells, tissues, and organisms

following xenobiotic exposure can be detected by monitoring metabolite variations and can be studied via a metabolomics approach. Metabolomics can provide a fast screening of a wide range of metabolites (biomarkers) that are related to well-defined pathways or processes, therefore giving mechanistic insight into NMs toxicity. The main techniques applied in metabolomic studies are liquid chromatography coupled with mass spectrometry and nuclear magnetic resonance spectroscopy (Lv et al. 2015).

Moreover, advanced microspectroscopy techniques are gaining importance for the evaluation of physiological and pathological events. For instance, infrared microspectroscopy, as a noninvasive and label-free technique, is considered a fast and informative multiscreening platform to study complex systems such as cells and tissues, allowing the characterization of the most important cellular components (proteins, lipids, nucleic acids, and carbohydrates), as well as their possible modifications, both at a compositional and a structural level. As an example, the gathered details obtained with this technique can provide information of membrane fluidity and composition, protein content and structure, and nucleic acid structural alterations. Thus, infrared microspectroscopy can overcome the limitations of classical methods, providing more accurate information for the toxicological evaluation of NMs and other xenobiotics (Bunaciu et al. 2014).

8.5 Challenges of Toxicological In Vitro Testing

As previously discussed, the physicochemical properties which make NMs interesting systems for the biomedical field are also correlated to their high reactivity and biological activity. Also, several experimental challenges, not only dependent on their physicochemical properties, emerge when studying NM-induced toxicity (Fig. 8.4). Thus, the safety assessment of NMs is often more complex than safety assessment of bulk materials.

Bulk material chemical and biological purity are relevant parameters for nanomedicine production and activity, as well as in hazard characterization. For instance, the presence of hazardous and bioactive contaminants, such as endotoxins, can induce inflammatory signaling mediators and other immunological responses, endotoxin shock, and even tissue injury (Azhdarzadeh et al. 2015). Thus, endotoxin content should be evaluated before immunogenicity studies. If the presence of endotoxins is detected, they can be removed by sterilization methods, such as filtration, autoclaving, or irradiation (Peters et al. 2017). However, these conventional techniques can interfere with and alter NMs physicochemical properties, and, like a cycle, affect their efficacy (Vetten et al. 2014). Therefore, in the pharmaceutical industry more advanced techniques for endotoxin removal such as several types of chromatography and ultrafiltration are commonly performed. For instance, affinity chromatography Polymyxin B columns are very effective for endotoxin removal due to their very high binding affinity for lipid A, which is one of the main components of LPS. However, these facts only reveal that there is still no efficient and

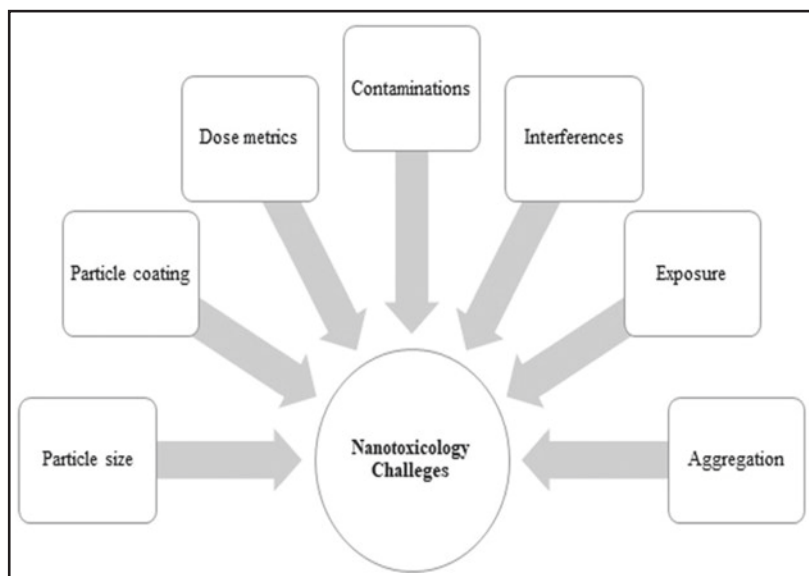


Fig. 8.4 Main challenges in nanotoxicology

widely established technique yet, so one simple way to prevent contamination is by choosing, promoting, and maintaining endotoxin-free NMs production processes (Peters et al. 2017; Du et al. 2017).

Also, unlike raw materials, NM preparations are often complex mixtures that may contain different populations that are present in various manners ranging from pristine forms to surface-modified systems, agglomerates, and/or aggregates (Benetti et al. 2014). The presence of these different populations impacts the mediation and promotion of the biological effects of NMs. Depending on its form, the same NM can show different safety and toxicological profiles (Stone et al. 2009; Teeguarden et al. 2007; Mahler et al. 2012).

The referred factors can also interfere with *in vitro* testing (Table 8.3), causing artifacts and unreliable results (Dobrovolskaia et al. 2016; Doak et al. 2009). That is one of the several reasons why recently research have been also focusing on overcoming the difficulties in the adaptation of *in vitro* common assays for conventional chemicals to evaluate NMs (Love et al. 2012; Monteiro-Riviere et al. 2009; Stone et al. 2009; Sharifi et al. 2012).

Most commonly, *in vitro* assays outputs are obtained by optical detection of specific molecular probes by absorbance, fluorescence, or luminescence analysis. However, it is also known that NMs can cause optical interferences as they may scatter or absorb light within these tests spectral range (Love et al. 2012; Stone et al. 2009; Dobrovolskaia et al. 2010, 2016; Doak et al. 2009; Powell et al. 2010; Oberdörster 2004). These type of interferences were observed in the testing of several nanosystems such as metallic and inorganic nanoparticles, as well as single-wall carbon nanotubes (Knuschke et al. 2013; Dobrovolskaia et al. 2016; Doak et al.

Table 8.3 Potential interferences in in vitro nanocarrier toxicity assays and possible troubleshooting

Potential interferences	Effects	Troubleshooting
Optical	Light scattering	Removal of nanomaterials before reading
	Increase of molecular probe absorption	
	Light absorbance	Removal of nanomaterials before reading or subtract nanomaterial absorbance as background when performing absorption spectroscopy
	Increase of molecular probe absorption	
	Decrease excitation light and emission spectrum of fluorescent probes	
Physical	Molecular probe adsorption	Removal of nanomaterials before performing the assay
	Decrease absorbance or fluorescence preventing enzymatic reactions due to reduction of end products and/or catalytic enzymes	
Physico-chemical	Nanomaterial reactivity	Removal of nanomaterials before performing the assay or if retained replace the assay
	Catalysis of molecular probes by nanomaterials	
	Dissolution	
	System interferences	Replace of cell culture medium with buffer or selection of the most inert cell culture components
	Catalysis of molecular probes by cell culture medium and serum	

Adapted from Ciappellano et al. (2016)

2009; Farrera and Fadeel 2015). Moreover, NMs can also cause result alterations by the direct interaction with assay components. As an example, they may interact and adsorb on their surface the molecular probes or cellular components, causing decreased absorption/emission spectra and less accurate measurements (Holder et al. 2012). Recent literature surveys reported that about 90% of published works do not reference the use of appropriate controls to avoid and/or detect NM interference (Ong et al. 2014). However, some studies reported the interaction of NMs with assay components or the detection and measurement of intrinsic NM fluorescence (Holder et al. 2012; Ong et al. 2014). Thus, the lack of appropriate controls is also a limitation to overcome and has contributed significantly to conflicting results on NM toxicological assessment reports. This also unveils the need for more specific and accurate standards in nanotoxicology.

Thus, to avoid these artifacts and interferences and to validate proper and adequate results, the performance of a primary screening of different in vitro assays with different outputs and endpoints, as well, and detection methods is recommended for the evaluation of NMs toxicity.

8.6 Conclusions and Future Perspectives

During the last decades, there has been a growing interest for medical applications of NMs opening a new perspective and offered new strategies toward the development of advanced and highly personalized treatments. However, NMs' unique physicochemical profiles and subsequent high reactivity can be also responsible for adverse effects of induction on humans. Thus, concern with proper evaluation of risks associated with NMs exposure has also risen. To minimize the risk, an accurate and proper analysis of efficacy and safety of newly developed NMs should be performed. Nonetheless, to date, NMs safety and toxicological testing has not been performed with a common and rational strategy.

In this chapter, we mainly described the commonly used approaches for in vitro nanotoxicology, highlighting advantages and limitations of these techniques and methodologies in order to unveil the challenges related the safety and toxicological evaluation of NMs. Different analytical methodologies are being used for nanotoxicity assessment purposes from conventional testing protocols to advanced analytical techniques, such as “omics” techniques. However, before performing in vitro testing on NMs, it is mandatory to execute a prior and extensive physicochemical profile characterization with a minimum set of features including chemical composition, size, shape, and surface properties. Moreover, NMs interferences within the assays should be also carefully evaluated aiming to select the best test or modify it if necessary.

This chapter was also dedicated only to in vitro testing because, to date, most of the advances and publications in the field of nanotoxicology focuses on the evaluation of biological responses at a cellular level. Nevertheless, in vitro study results lack further corroboration with in vivo models, in order to provide a complete understanding on the toxicological profile of NMs in complex living systems.

Globally, despite the progress on the field, there is still an imperative need for new advances and improvements in analytical techniques and protocols with specificity for nanotoxicological assessment. This demand should consider overall influential factors and limitations of the current assessment protocols, and the development of appropriate controls and complementary studies. All these factors will contribute to establish an improved and standardized evaluation resulting in more reliable information on potential risks of NMs.

Acknowledgments The authors would like to thank the financial support received from Portuguese Science and Technology Foundation (FCT/MCT) and from European Funds (PRODER/COMPETE) for the projects M-ERA-NET/0004/2015 and UIDB/04469/2020 (strategic fund), and co-financed by FEDER, under the Partnership Agreement PT2020. MCT wishes to acknowledge FCT and Dendropharma – *Investigação E Serviços De Intervenção Farmacêutica, Sociedade Unipessoal Lda.* for the individual fellowship (PD/BDE/135086/2017).

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Chapter 9

Impact of Nanomaterials on the Food Chain



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Abstract

Background/Issues

The applications of nanoscience and nanotechnology in agriculture and food sector are relatively recent. Substantial acquisitions due to the effects of nanomaterials in these fields have expanded their usage. Intriguingly, nanomaterials demonstrate several biological complications in studies. Hence, the recent entrance of this technology into the human food chain has arisen concerns.

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Major Advances

Here we review the possibility of naturally occurring nanomaterials existence in food. The routes of engineered nanomaterials entry through the ecosystem to the plant and animal food and their bioaccumulation and biomagnification are delineated. Despite the limitations in nanomaterial toxicity assessment, awareness about nanomaterial's movement through different trophic levels and their effects on food animals and plants might help the risk analysis of these particles in the food chain.

Keywords Nanomaterial · Food chain · Ecosystem · Impact · Toxicity

9.1 Preface

After the first industrial revolution in the mid-eighteenth century, nothing could mark a major turning point in the global economic empire as much as nano-industry by now. Industries invested billions of dollars in nanotechnology innovations, and its trading value is assumed to reach approximately \$3 trillion by the year 2020 (Roco et al. 2011). Nanotechnology seems to be the magic lamp, so it has the power to prevail over all of the barriers and make everything possible in all industries including the food production scope. European Food Safety Authority (EFSA) defined various nanotech-related products in different food sectors (Hardy et al. 2018). According to the nano-database (<http://nanodb.dk/>), 3037 nanotechnology-derived food products are available on the market for customers in 2018; therefore, it is not surprising that in countries with hi-technologies, it is estimated that billions of nanoparticles have been consumed daily by people, and this number will be increased obviously in near future (Rompelberg et al. 2016).

As it is shown in Fig. 9.1, the wide range of engineered nanomaterials or man-made nanomaterials in food is a part of the story of human's exposure to nanoparticles. It is well established that in some food manufacturing processes, especially those which used pressure or laser in some steps, new endogenous molecular structures have been created accidentally as food-borne or process-generated nanoparticles (Brody et al. 2008). Results of a study in 2018 have approved the presence of 5 nm fluorescent nanoparticles in the most worldwide popular beverages (Li et al. 2018). Application of intentionally manufactured nanomaterials in plant and animal husbandry is one of the major causes of increasing food-related risks for consumers, for example, hundreds of crop protectants and veterinary medicines. Unfortunately, existing data on their final destination in the environment is still scarce (Kah et al. 2013; Dimkpa et al. 2013; Giannousi et al. 2013). Surprisingly every year thousands of tons of nanoscale materials have been drained globally into the landfills and water.

The entrance of nanomaterials into the water, soil, and atmosphere can have important consequences for animal and plant health through distribution in the main structural components of the plant and microbial community in the soil (Rico et al. 2011; Ma et al. 2010). As the engineered nanomaterials enter into the ecosystem, the biological entities and inorganic substances stochastically transform them and

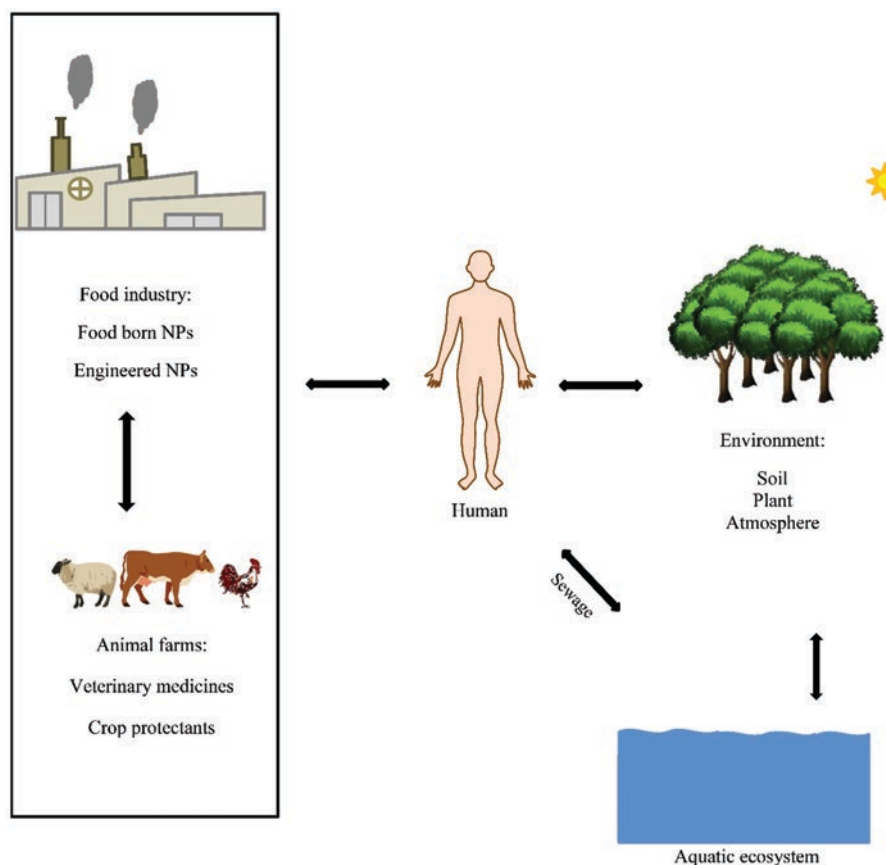


Fig. 9.1 Uncontrolled nanoparticles distribution in the environment

modify their properties that affect their fate (Glenn and Klaine 2013; Hernandez-Viezcas et al. 2013; Sun et al. 2009; Lowry et al. 2010).

They also interact with food chain organisms at lower trophic levels and/or upper ones (Keller and Lazareva 2013). The emerging information on trophic transfer assessment has corroborated that at least two trophic steps contaminated with nanomaterials (Mortimer et al. 2016; Kim et al. 2016; Chae and An 2016). Findings in 2017 showed that behavioral disorders of a fish are connected to plastic nanoparticles in its brain as a top consumer; however, we could not completely follow up details of nanomaterials journey in the body (Mattsson et al. 2017).

When nanomaterials have been taken up by living cells, they can be accumulated within cell membrane and various parts of cytoplasm or they can cling to a set of different components within surrounding environment like gastrointestinal fluids, food matrix, and even microbiota (Lai et al. 2009; Crater and Carrier 2010; Nel et al. 2006; Oberdorster et al. 2005; Li et al. 2018). Protein conjugation with engineered nanomaterials may increase their entrance to the cells (John et al. 2001;

Pante and Kann 2002; John et al. 2003). The roadmap of passing particles through the intestinal wall and reaching to a biological fluid has been included multiple unforeseen challenges because tight junctions pore size are 0.3–1.0 nm (des Rieux et al. 2006).

Several studies have addressed the various biological toxicity of engineered nanomaterials, which clearly have shown their ability to initiate irreversible biochemical functions, such as oxidative stress, release toxic ions, mitochondrial perturbation, metabolomic and proteomic changes, which ultimately connected to other complications like altering cell cycle regulation, apoptosis, DNA damage, and inflammatory status (Chen et al. 2007; Peters et al. 2012; Li et al. 2018; Xie et al. 2018; Lindeque et al. 2018; Møller et al. 2017). Literature analysis revealed that induction of reactive oxygen species (ROS) is behind the most pathophysiologic basis of the nanoparticle tissue damage (Fig. 9.2) (Donaldson et al. 2009). Application of man-made nanomaterials in various Hi-Tech agri-food industries is growing due to the popularity of these tiny materials. There is a serious alarm regarding the day-to-day increment of worldwide human exposure through the different pathways, especially the food chain (Kaluza et al. 2009).

9.2 Naturally Occurring Nanomaterials in Food

The natural processes that produce nanoparticles can be photochemical reactions, forest fires, volcanic eruptions, simple erosions, and even by plants and animals (Buzea et al. 2007). Naturally, through the food chain, all foods including plants and animals that have been used for centuries include nanomaterials (Magnuson et al. 2011). Natural organic molecules in foods such as proteins, carbohydrates, and fats have different sizes from large polymers to simple nanoscale molecules (Magnuson et al. 2011; Raynes et al. 2014; Sun et al. 2014; Bouhallab et al. 2017). The main components of milk with dimensions of about 0.5–300 nm, such as casein micelles, whey proteins, and lactose, are natural nanomaterials (Magnuson et al. 2011; Sun et al. 2014). The casein micelle, as one of the abundant proteins in milk, measures between 100 and 200 nm (Bouhallab et al. 2017). Milk fat globules, β -lactoglobulin, α -lactalbumin, ovalbumin, lysozyme, ovotransferrin, avidin, as well as myofibrillar proteins are among the natural nano-sized organic compounds in milk, meat, and egg (Bouhalla et al. 2017; Morris 2010; Peters et al. 2016; Brownlow et al. 1997; Majorek et al. 2012). In food nanotechnology, many of these natural nano-sized molecules play important roles in the functional and nutritional properties of foods (Rogers 2016; Sekhon 2010).

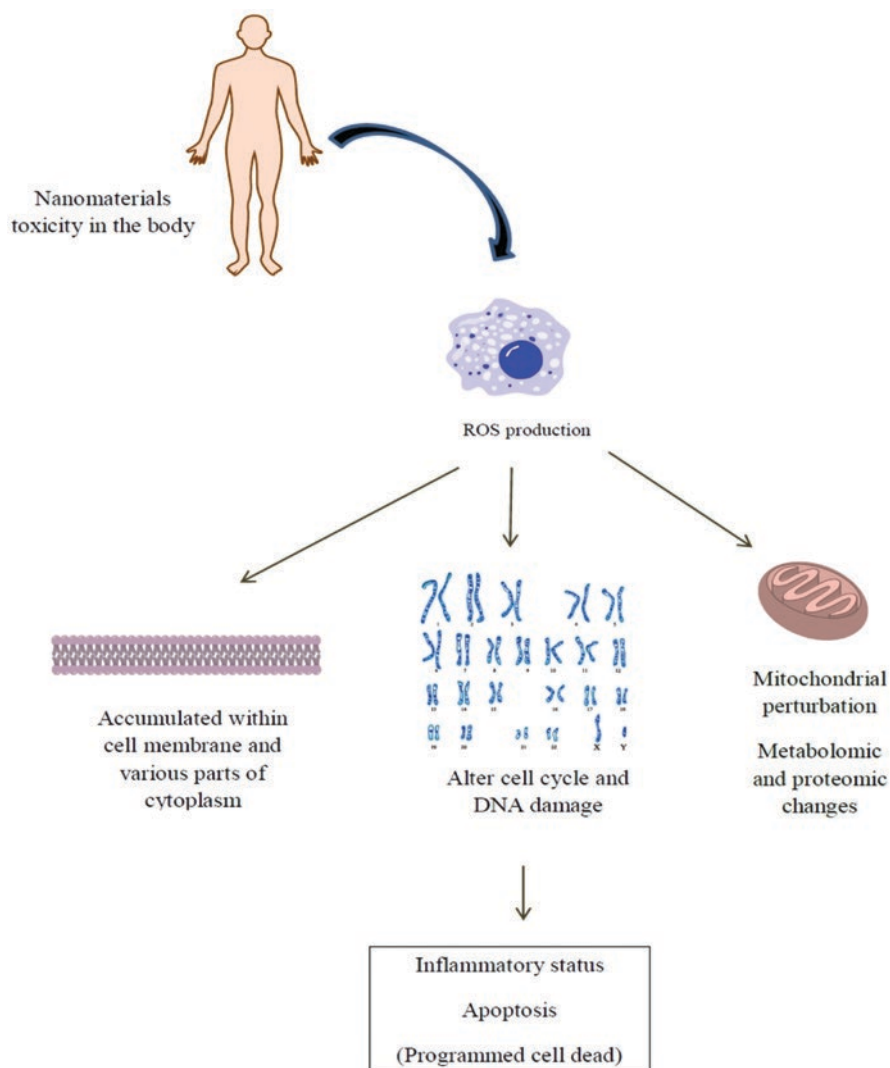


Fig. 9.2 Toxicity of reactive oxygen species (ROS) in the cell

9.3 Contamination of Food-Associated Ecosystems with Nanomaterials

Advances in nanotechnology and extensive nanoparticles usage can transfer them directly or indirectly into the environment. The food chain can be contaminated with nanomaterials through a variety of sources in the ecosystem (Yah et al. 2012). Therefore, the human body is being polluted by nanomaterials directly via water,

soil, and air, or indirectly through the consumption of foods of plant and animal origin (Ingale and Chaudhari 2018).

Plants interact directly with the environmental compartments: soil, water, and atmosphere, all of which can be routes of engineered nanomaterials distribution. Furthermore, nanotechnology contribution in agriculture is increasing day by day to achieve a higher and more stable yield of food grains based on optimizing water and nutrient supply (Scott and Chen 2013). Hence, plants are also subject to extensive human manipulation and are potentially subject to engineered nanomaterials exposure from multiple sources. Nanomaterials destined for applications in biotechnology or nano-agriculture are sometimes designed for uptake by plants, and therefore their transport and bioaccumulation through the food chain is plausible (Miralles et al. 2012; Remédios and Bastos 2012; Rico et al. 2011). Some of the applied nanomaterials in agriculture are single-walled or multi-walled carbon nanotubes and metal-based nanoparticles (Dubey and Mailapalli 2016; Khodakovskaya et al. 2012).

Synthesis, application, and incineration of products containing nanomaterials in the food industry not only directly endangers food industry workers but also facilitate their leakage into the environment via different routes such as atmospheric pollution (Bakand et al. 2012; Dasgupta and Ranjan 2018). Nanomaterials in the atmosphere can deposit on the leaves or other aerial parts of plants, aggregating on tissue surfaces and penetrating through stomatal pathways (Navarro et al. 2008; Zhu et al. 2008).

Nanoparticles (such as Zn, TiO_2 , and SiO_2) may directly or indirectly pollute soil through runoff and biosolids, sewage wastes, and plant residues (Ingale and Chaudhari 2018). Those adsorbed on soil and sediments can interact with plant roots (Navarro et al. 2008; Zhu et al. 2008).

Water involves nanomaterials by direct contamination of water reservoirs or through the water and sewage purification and remediation, agricultural-lands biosolids application or disposal into the landfills. In this regard, plants, terrestrial and aquatic food animals, and human are the main victims (Ingale and Chaudhari 2018; Magnuson 2009; Klaine et al. 2008). Disposal of waste from nanoparticle production plants may accidentally enter the environment and contaminate soil and surface waters (rivers, ponds, or reservoirs, mostly regarded as drinkable waters) or underground waters via wind or rain (Zhu et al. 2012). Moreover, using nanoparticle-contaminated landfill leachates and sewage sludge for soil fertilization is regarded as the main route of contamination of underground waters and soil (Klaine et al. 2008).

9.4 Uptake, Bioaccumulation, and Biomagnification of Nanomaterials in Food

Recently, nanotechnology is incorporated into the agricultural and food industries. As mentioned before, the current increase in the production and application of nanomaterials has exposed plants to these materials; therefore, plants, as the first producers in consumers' food pyramid, are regarded as the main route of nanoparticles entry into the food chain.

Despite the exposure of plants to the undesirable environmental nanomaterial pollutants, the application of nanomaterials in the agricultural industry has gained credit at least in laboratory scales. Nanomaterials, especially nano-fertilizers or pesticides, successfully helped in increasing germination, plant growth and crop quality, photosynthesis enhancement, plant nutrient use efficiency and higher water uptake inside seeds (Khodakovskaya et al. 2012; Harrison 1996; Nair et al. 2010; Giraldo et al. 2014; Kottegoda et al. 2011; Milani et al. 2012; Wilson et al. 2008; Tripathi et al. 2011).

Accumulation and absorption of nanomaterials by plants may affect the food chain and harm human health. The uptake, accumulation, and transformation of nanomaterials in food crops are yet ambiguous. Notably, few plant studies have revealed that the nanomaterials characteristics, plant species, and even experimental conditions can significantly influence on nanomaterials–plant interactions (Rico et al. 2011; Monica and Cremonini 2009).

It has been shown that some plants absorb nanomaterials using stomata of leaves, which are in contact with air and foliar sprays and also their roots. They store them in their tissues and transfer them to other parts (Ingale and Chaudhari 2018; Anjum et al. 2016; Dhoke et al. 2013). Following the root uptake and epidermal cell penetration of nanomaterials, further transportation of nanomaterial to the xylem is necessary for their circulation. It seems that three routes are involved in nanomaterial transportation: (i) cell wall pores, (ii) the apoplastic pathway, or (iii) 40-nm plasmodesmata channels that connect adjacent plant cells, which is called the symplastic pathway (Luttge 1971; Tilney et al. 1991). The translocation of nanomaterials from the roots to the plant aerial parts has been studied. Intra- and/or extracellular movement of nanomaterials through tissues brings them to the xylem (Fig. 9.3). Passage of nanomaterials across the root might not be as simple as we think; it can be affected by the endodermis cell wall between cortex and stele called Casparian strip in the root that prevents the apoplastic transport of external materials (Luttge 1971; Seeger et al. 2009). If this cell-wall incrustation was continuous, the symplastic pathway is the only way of crossing nanomaterial from the plasma membrane and reaching the stele; however, root apex or the damaged endodermal cells are the probable substitute paths for nanomaterials (Tester and Leigh 2001; Nowack and Bucheli 2007; Wang et al. 2012). Upon getting into the vascular system, the transpiration stream could translocate nanomaterials to the aerial parts of the plant (Lin et al. 2009).

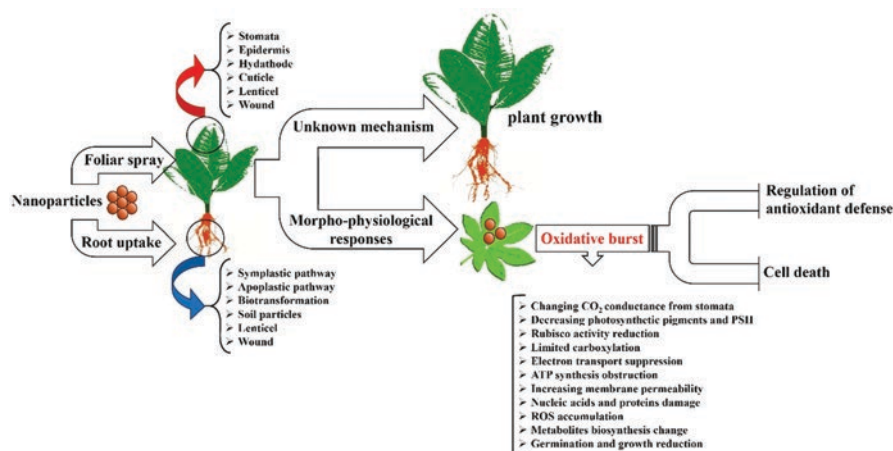


Fig. 9.3 Uptake and translocation pathways of nanomaterials and their presumptive effects on plants

The accumulated nanomaterials in plant tissues are eaten by the human or enter the human food chain after animals are exposed to nanoparticle-contaminated plants. For instance, there are reports of TiO₂ nanoparticles transfer from soil to the vegetables (Servin et al. 2013).

Food animals might receive nanomaterials through the use of contaminated food or water. Usual routes include the entry of nanomaterial-contaminated agricultural products during agricultural operations into the animal food chain, veterinary medications containing nanomaterials such as certain vaccines, the application of live-stock nano-nutritional and growth-promoting supplements, and also the antimicrobial nanomaterials in abattoirs and meat processing plants. After contamination, they may be stored in animal tissues consumed by the human (Cushen et al. 2012; Ingale and Chaudhari 2018; Kim et al. 2018).

9.5 Food Industry Welcomes Nanomaterials

Obviously nanotechnologies open up a special place in the food industry for developing several novel products, including nanoparticles, such as liposomes, micelles, biopolymeric nanoparticles nanoemulsions, as well as the production of nanosensors and nanotracers, which are aimed at ensuring food safety (Ligler et al. 2003; Nasongkla et al. 2006).

The addition of nano-sized supplements to the livestock feed or water may help to improve the quality of the meat or eggs and also the production cycle. For instance, a study showed that the addition of chromium nanoparticles to the bird feed can affect the protein content of breast and thigh muscle and decrease cholesterol (Hill and Li 2017). Animal health can be improved using bioactive compounds

and nutrients added to their diet with the intervention of nanotechnology (Ötles and Şahyar 2017). Certain nanoparticles are applied as feed additives (Gangadoo et al. 2016; Gopi et al. 2017; Peters et al. 2016; Zhao et al. 2014). Some nanoparticles help increase the absorption of nutrients, such as selenium and iron, in order to improve digestion in sheep (Pelyhe and Mézes 2013). Furthermore, selenium plays a significant role as an antioxidant and has positive effects on the growth, fertility, and immune system of farm animals. These nanoparticles are highly bioavailable nutrients with applications in broilers, livestock, and goats (Peters et al. 2016; Pelyhe and Mézes 2013). In some cases, nanoparticles can be used to enhance the bioactivity in functional foods (Chau et al. 2007). They can be used to promoting the efficiency of bioactive compounds such as omega-3 acid from salmon oil by increasing their solubility, bioavailability, and stability during processing, storage, and distribution (Chen et al. 2006). For instance, polymeric nanoparticles are suitable for the bioactive compounds encapsulation (e.g., vitamins and flavonoids) and releasing them in acidic environments (i.e., stomach) (Pool et al. 2012). Bioactive packaging materials can preserve the bioactive compounds, such as prebiotics, probiotics, encapsulated vitamins, or bioavailable flavonoids, in an optimum condition till their controlled release into the food product. Carrageenan, alginate, gelatin, chitosan, polylactic acid, and polyglycolic acid are approved food additives for the nano-encapsulation (Lopez-Rubio et al. 2006). Protecting antimicrobial agents by nano-encapsulation and increasing their delivery improves cell absorption and their antimicrobial activity (Blanco-Padilla et al. 2014). The nano-encapsulation technology is also used in aquatic feed production to provide nutrients such as destructible fatty acids or fat-soluble vitamins, which are not sufficiently soluble and absorbed in the fish intestine (Handy et al. 2011).

Today, the application of nanocapsules containing additives is also expanding. Self-assembled nanotubes made of hydrolyzed milk protein and α -lactalbumin in the form of nanocapsules can carry nutrients, additives, and supplements (Chaudhry et al. 2008).

Food packaging is part of the food industry. Despite the conventional food storage methods and different food packages that can ensure the quality of food, nanoscience with its various capacities opens its way into the food packaging technology (Scott and Chen 2013; Magnuson et al. 2011; Baeumner 2004; Su et al. 2013; Hamad et al. 2018). Nanomaterials and bionanocomposites, as hybrid nanostructured materials, in food industry enhances thermal, mechanical, and gas exchange properties of food packages (Darder et al. 2007). In this way, even the sensory and physicochemical properties of fruits such as strawberry could be preserved using nano-packages (Yang et al. 2010). Furthermore, freshness maintenance of apple slices exposed to nanomaterials in comparison to low-density polyethylene (LDPE) along with the anti-browning activity of these tiny materials are some other examples (Li et al. 2011; Zambrano-Zaragoza et al. 2014; Zhou et al. 2011; Ekielski et al. 2015). Nanomaterial application in food packaging not only preserve food quality and safety but also reduces the usage of plastic bags (Suyatma et al. 2004; Sorrentino et al. 2007).

Smart and active packaging and the usage of natural polymers (based on bio-nanocomposites) are another aspects for nanomaterial application to increase the quality, safety, and longevity of food products (Cushen et al. 2012). Prevention of microbial spoilage, chemical deterioration, and the improvement of sensorial and nutritional properties of food are the main key rationales. Active food packaging also can alert consumers when the content is spoiled. As the nanosensors ensure the quality of the product by detecting microbes, toxins, and pollutants in food packages, the active package releases compounds such as antimicrobials, flavors, colors, or supplements to the food (Neethirajan and Jayas 2011; Chaudhry et al. 2008).

Numerous studies showed that the surface of foods such as cheese and minced meat that are prone to spoilage could be covered and ultimately protected through the packaging prepared from silver, zinc, and other antimicrobial nanoparticles (Buonocore et al. 2005; Véronique 2008; Ramachandraiah et al. 2015; Abdou et al. 2012). In this way, lots of organisms such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, yeasts or molds, and even resistant spores could be inhibited. These nanomaterials might apply in different forms such as pads, films, or even internal walls of milking devices for the prevention of microbial growth (Panea et al. 2014; Karimi et al. 2018; Cushen et al. 2012; Yildiz and Pala 2012; Akbar and Anal 2014; Arfat et al. 2016). Nanofilms are coatings on the product with an embedded bioactive nanomaterial, which also affect the loss of aroma, taste, moisture, and gaseous exchange, thereby maintaining the food quality and increasing the shelf life (Turan et al. 2018).

Nanotechnology has produced oxygen scavengers and moisture-absorbing leaves for products such as fresh meat, chicken, and fish. Nano-packages with the ability to reduce the entry of oxygen and other gases and moisture exhaustion can prevent food spoilage (Rivett and Speer 2009). Furthermore, TiO_2 nanoparticles that control microbial growth and protect food packaging against UV rays are used in a series of packages to maintain the quality and safety of some products such as fresh cheese, yogurt, and meat (Karimi et al. 2018; Cushen et al. 2012).

Notably, there are serious concerns about nanomaterial increasing usage in farm animals and food industry because there is little information on nanoparticle migration from the packaging or coatings into the food and also the impact of these nanomaterials on the human health (Bumbudsanpharoke and Ko 2015).

9.6 Nanomaterials as Regulatory Tools in Agri-Food Systems

Different types of nanomaterials have been produced at the range of 1–100 nm. Hence, nanobiotechnology has revolutionized the various sectors of the agri-food industry through the designation of engineered nanomaterials, instruments, and systems (Livnah et al. 1993; Thulasi et al. 2013; Gangadoo et al. 2016; Martirosyan and Schneider 2014). For instance, in food safety and quality sector, nutrient deficiency and toxicity along with animal and plant disease detection are being provided by nanosensors through measuring any state of nutrients.

Nanosensors improve production in agriculture with increasing efficiency of soil suppliers such as minimum loss of input like irrigation, pesticide, and fertilizers (Scott and Chen 2013). The most used nanosensors in agriculture are (i) bionanosensors and (ii) electrical-nanosensors. In agriculture and food arena, biosensors are manufactured for the exact assessment of microcystin toxicity as the hepatotoxins produced by cyanobacteria that put the agriculture, animal's and human's health at risk (Singh et al. 2012). Accurate time-based information including food and environment pesticide detection is achievable by the wireless nanosensors (Dubey and Mailapalli 2016). Carbon nanotube-based electrochemical sensor with deposited gold nanoparticle was designed to detect triazophos, which is an insecticide in post-harvest vegetables (Li et al. 2012). Gold and silver nanoparticles also used in a biosensor to detect organophosphorus pesticide level in the environment and post-harvest food (Simonian et al. 2005; Wu et al. 2011).

Monitoring food safety and quality is also important in food production and processing industries while identifying food spoilage at early stages is vital for consumers. Hence, nanotechnology can play its role in providing advanced quick diagnostic sensors for the food spoilage detection and therefore controlling both food safety and quality (Ingale and Chaudhari 2018; Bhattacharya et al. 2007; Kumar et al. 2017). Nanosensors have a lot of potentials to accelerate the detection, identification, and determination of pathogens, spoiled material, chemical agents such as xanthine and hypoxanthine, mycotoxins, allergic proteins, and food freshness. They also help with the detection of any food color and gaseous changes due to the food spoilage. Therefore, nanosensors are significantly effective in many agri-food sections (Ramachandraiah et al. 2015; Mao et al. 2006; Hamad et al. 2018; Karimi et al. 2018; Attia et al. 2018). Notably, the higher sensitivity and selectivity of nanosensors in the food industry makes them more efficient than the conventional sensor systems (Hamad et al. 2018).

Produced chemicals throughout food spoilage can be detected via the package nanosensors, which act as electronic tongues or noses (García et al. 2006). Sensitive, tiny, and widespread nanosensors based on microfluidics devices can also be applied for fast pathogen detection in real time. These sensors need only microliters of sample volumes for the detection of compounds (Baeumner 2004; Mabeck and Malliaras 2006; Vo-Dinh et al. 2001). Silicon-based microfluidic systems have proven popular in the so-called laboratory-on-a-chip technology (Tay 2002).

Devices with the “nanoelectromechanical systems” (NEMS) technology are already in use for the food analysis and might serve as developing tools in food preservation. They can control the storage environment. NEMS could be used in food quality-control devices because they consist of advanced transducers for specific chemical and biochemical signal detection. The use of so-called micro- and nanotechnologies (MNTs) has advantages for food technology. Some of the advantages are low costs, portable instrumentation with quick response, and smart communication through various frequency levels. MNTs in the field of food safety and quality can discover adulterations in packaging and storage conditions (Ravichandran 2010).

Nanocantilevers are another class of biosensors. Detection of biological-binding interactions between antigen-antibody, enzyme-substrate, etc. through physical and/or electro-mechanical signaling is the basis of these biosensors (Hall 2002). Their tiny parts of silicon-based materials make them capable of recognizing proteins and detecting pathogenic microorganisms (Kumar 2007). Molecular interaction studies and detection of food chemicals such as antibiotics and toxins are already indebted to the nanocantilever devices (Ramírez-Frómata 2006). A European Union-funded project called Bio-Finger developed a nanocantilever device that could be used to detect pathogens in food and water based on the sensing of ligand-receptor interactions (Jain and Jain 2008).

Nanotracers and nanomonitors are another monitoring tool to detect and measure the concentration, surface, and size of nanoparticles. Nanotracer usage in air quality monitoring, environmental monitoring, and the nanoparticle exposure assessment leads to the food safety, food chain security, and human health (Diallo and Brinker 2011; Marra et al. 2010).

9.7 Nanomaterial Toxicity in Food Animals and Plants

Although the application of nano-based sciences has pushed various industries forward quickly, the development of this technology may have adverse consequences on the environment and different creatures.

The lengthy food chain and the simplicity of nanomaterial motion have some negative effects on water, soil, vegetation, aquatic life, and human. Nano-fertilizers could be detrimental to the beneficial microorganisms and fertility of the soil and moreover changes its structure and texture. The size and concentration of nanoparticles are the principal agents for eco-toxicity (Raliya et al. 2013).

Despite the plant growth stimulation at low doses of nanomaterials, they mostly prevent the growth at high levels (Zheng et al. 2005). The high concentration of nanomaterials leads to oxidative damage by the stimulation of ROS production and accumulation, which inhibits photosynthesis, promotes stomatal closure, and alters enzyme activities. Electron leakage, lipid peroxidation, and subsequent membrane damage, as well as nucleic acids and proteins damage, make ROS a threat to the cells (Fig. 9.3) (Li et al. 2003; Donaldson et al. 2004).

The responses of plants to the phytotoxicity of nanomaterials not only depend on the plant species, genotype, age, and stage of development but also they are influenced by the concentration and size of nanoparticles. To assess the exposure effects of nanomaterials on plants, phytotoxicity evaluations are being executed mostly during (i) plant germination or (ii) seedling elongation (Lin and Xing 2007; Ma et al. 2010). Due to the augmented reactivity and toxicity of smaller-sized nanoparticles at especially elevated doses, the plant photosynthesis and respiration processes are drastically being affected (Navarro et al. 2008). The phytotoxicity impact of Al_2O_3 nanoparticle caused a reduction in *Zea mays* root. Alumina nanoparticles enhanced root growth of *Raphanus raphanistrum* and *Brassica napus* while

prevented root length of *Lactuca sativa* and *Lolium sp.* but had no significant effect on *Cucumis sativus* (Lin and Xing 2007; Miralles et al. 2012).

The study of nanoparticles behavior at different sizes and different concentrations in soil, water, and the plants is necessary to understand their agro-ecological toxicity. In animals, nanoparticles such as Ag nanoparticles can enter the cell through endocytosis or diffusion. Furthermore, these nanoparticles can affect mucous proteins function and cause mitochondrial disruption, DNA damage, and chromosome disorders. Consistently, other effects such as mucosal diseases, inhibition of antioxidant enzymes, oxidative stress, inflammation, and apoptosis (programmed cell death) are also being observed (Thulasi et al. 2013; Karimi et al. 2018).

The entrance of nanoparticles to the aquatic ecosystems has tremendous effects on the environment and organisms. The fish are good indicators of environmental health. Based on available reports, fish and daphnids are among the most sensitive aquatic animals to nanoparticles (Asghar et al. 2015). In general, nanoparticle toxicity is mostly reported in the primary stages of fish life. Poor data are available on the effect of nanoparticles on the physiologic behavior of aquatic organisms. Fish skin and gills are the most reported sites for nanoparticles negative effects, while histopathological changes in the intestine, liver, and kidney, as well as the accumulation of nanoparticles in tissues, are being discovered. ROS production, inflammation, oxidative stress, lipid peroxidation, cell and vascular damage, alteration of enzymes activity, the fish larval abnormalities and hatching retardation, morphological changes of tissues and tumor formation, and growth have been observed in various fishes such as rainbow trout, catfish, and zebrafish (Karimi et al. 2018; Cushen et al. 2012; Asghar et al. 2015; Zhu et al. 2012).

9.8 Conclusions and Outlooks

Although the incorporation of nanotechnology in the industries is a significant step toward the human civilization glow, we should not underestimate the effect of a huge amount of unsafe manufactured nanomaterials in the terrestrial and aquatic food chain. Therefore, it is necessary to gather and evaluate baseline information on engineered nanoparticle hazard assessment and also it is crucial to unravel the biological outcomes of nanoparticle consumption. To achieve these aims, we can apply computer networks for classifying and hazard ranking of nanomaterials. However, legislative agencies face challenges for publishing worldwide accepted rules or guidelines for the assessment of nanoparticle hazards and potential risks on the food chain. Detection, quantification, and the precise analysis of different nanomaterials in foods and their environments along with the long-term biokinetics information in a human model are the most shackles in this road.

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Chapter 10

Phytoresponse to Nanoparticle Exposure



Vineet Kumar, Praveen Guleria, and Shivendu Ranjan

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Abstract Nanoparticles (NPs) can induce toxic effect due to their small size, surface reactivity, and chemical nature. They have been used to improve the efficiency and quality of various industrial products. The release of nanoparticles from such nanoproducts can bring them in contact with the primary producer, plants. They can have positive as well as negative effect on the plants. NPs have positive effect on seed germination, plant growth, and development mainly due to increased water and nutrient uptake, photosynthesis, and secondary metabolism by regulating genetic material and changing cell morphology. The negative effect on plant growth and development on NP exposure is mainly due to reduced photosynthesis, altered genetic material expression, and oxidative or abiotic stress induced by them. One or more factors can act simultaneously or individually to induce positive or negative

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V. Kumar et al. (eds.), *Nanotoxicology and Nanoecotoxicology Vol. 1*, Environmental Chemistry for a Sustainable World 59, https://doi.org/10.1007/978-3-030-63241-0_10

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effect on plants. Hence, the overall prediction of positive and negative effects of even same type of nanoparticles is difficult. Further, some NPs such as CuO, Pb, CeO₂, QDs, TiO₂, Au, Ag, and La₂O₃ NPs can breach the trophic levels. They have the tendency to transfer through trophic levels to reach the highest consumer level of the food chain. So, precise and thorough phytotoxicity evaluation of nanoparticles is urgently required on a case-to-case basis before their industrial-scale production and application.

Keywords Nanoparticles · Plant growth · Root growth · Photosynthesis · Seed germination · Plant biomass

10.1 Introduction

Nanoparticles (NPs) possess distinct physical and chemical properties due to their unique small size, shape, and surface chemistry (Roduner 2006; Handy et al. 2008; Kumar and Yadav 2009; Stampoulis et al. 2009). Interestingly, NPs possess higher number of surface atoms due to more surface area than their bulk counterparts. So, the surface reactivity of the material in the nanometer range is more than in the bulk form (Karakoti et al. 2006; Auffan et al. 2009). Hence, NPs have superior catalytic activity than their usual bulk counterparts (Reier et al. 2012; Schauermaun et al. 2013). However, surface reactivity of NPs is also directly related to their toxicity behavior. Therefore, surface reactivity may be beneficial for catalytic applications, while the same key feature may impart toxicity to living organisms (Huang et al. 2010; Duffin et al. 2007; Nel et al. 2007). Yet, it is interesting to evidence the increasing demand for NP-based products on a day-by-day basis, when the market is already occupied with NP-containing products.

Applications of NPs in daily-use products mean their uninterrupted exposure to the environment, plants, animals, and humans (Hristozov and Malsch 2009). The toxic effect of NPs on animals is well studied as compared to plants (Kumar et al. 2012; Kumar et al. 2014). Reports document the positive as well as negative effects of NPs on plant health, growth, and development (Cushen et al. 2012; Lee et al. 2013; Duncan and Pillai 2015). The number of studies reporting toxicity evaluation of NPs on plants has increased in the last 5 years. The present article thus discusses the interaction of NPs with plants in detail, with special focus on the mechanism of phytostimulatory and phytotoxic effects of NPs.

10.2 Plant–NP Interactions

Plants are the primary producers and first footstep of all (terrestrial) food chains and food webs. Just like nutrients, plants can uptake NPs from the soil (Rico et al. 2011a, b). Plants may be directly exposed to NPs by the use of NP-based agricultural

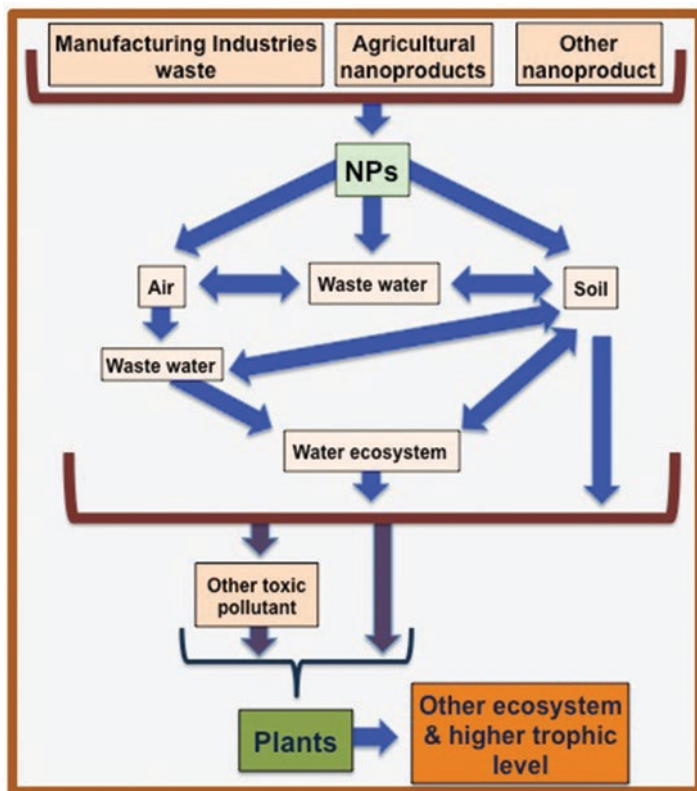


Fig. 10.1 Schematic presentation showing routes of NP contamination that ultimately affect plants

products and/or indirectly by means of industrial as well as household wastes containing NP-based materials (Fig. 10.1). NPs are released from household products of daily use (Fig. 10.2). Since plants are the prime food source for animals, their interaction with NPs is crucial. Plants are thus important biological indicators of risk associated with NP exposure (Ma et al. 2010; Rico et al. 2011a, b; Dietz and Herth 2011). NPs can enter plant cells by various routes as depicted in Fig. 10.3. Further after entering, the NPs can have no effect, positive or negative, on the plants.

10.2.1 Plant–NP Interaction: No Effect/Positive Effect

The interaction of NPs with plants may or may not affect plant health, growth, and development. In this section, positive or no effect of NPs on plants is discussed (Fig. 10.4). These NP–plant interactions are jointly addressed as beneficial or non-toxic. Some types of NPs possess plant growth-enhancing properties, whereas some are completely nonresponsive in this respect. NPs induce plant growth directly or

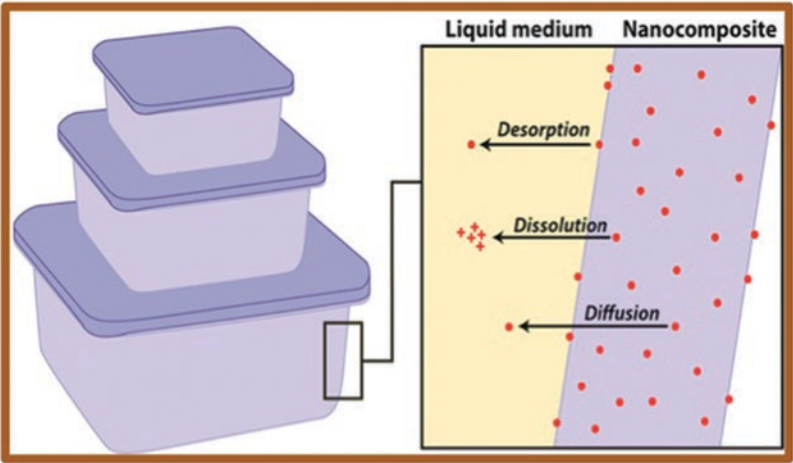


Fig. 10.2 Schematic presentation showing release of NPs from nanocomposite material used for making storage containers. “Reprinted with permission from (Duncan and Pillai 2015). Copyright (2015) American Chemical Society”

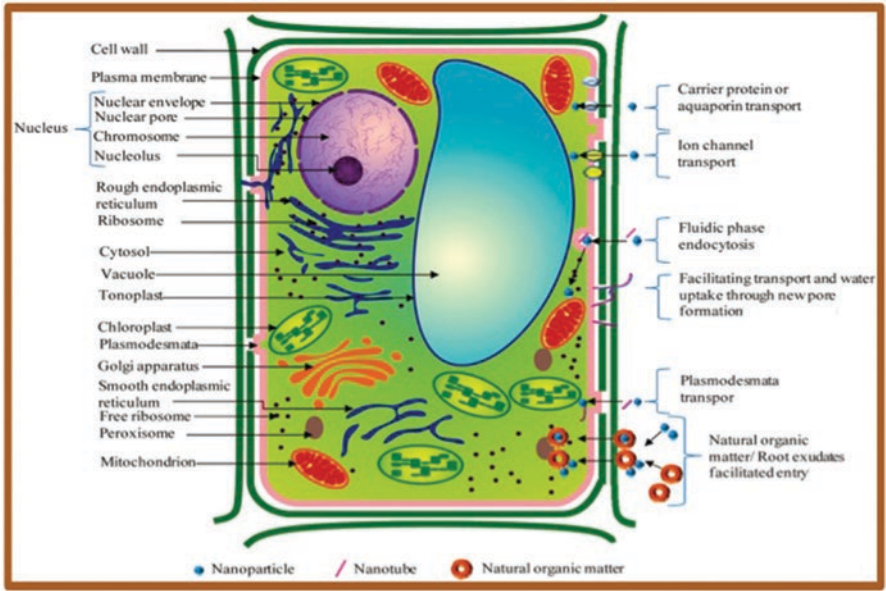


Fig. 10.3 Probable modes of cellular uptake of the nanoparticles in a plant cell. “Reprinted with permission from (Rico et al. 2011a, b). Copyright (2015) Springer”

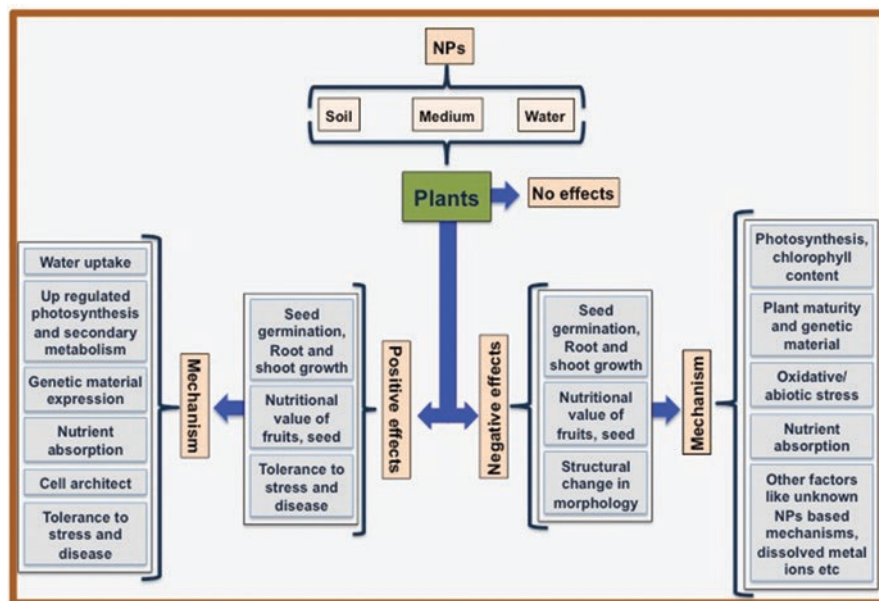


Fig. 10.4 Schematic presentation of effects and mechanism of NP action on plants

indirectly by influencing their growth and yield or by providing protection to plants against pathogens/phytotoxins/pesticide accumulations.

No Effect of NP Exposure on the Plants

Poly-3-aminobenzenesulfonic acid-functionalized single-walled carbon nanotubes (SWCNTs) as well as nonfunctionalized SWCNTs have no effect on the root length of cabbage and carrot plants (Canas et al. 2008). Likewise, multiwalled carbon nanotubes (MWCNTs), silver (Ag), copper (Cu), zinc oxide (ZnO), and silicon (Si) NPs has no influence on the seed germination of *Cucurbita pepo* plants (Stampoulis et al. 2009). Titanium dioxide (TiO₂) NPs have no effect on the water uptake efficiency, transpiration rate, and vegetative growth parameters of willow plants (Seeger et al. 2009). Undoped and nitrogen-doped TiO₂ NPs also do not affect the growth of maize and soybean plants. However, the same NPs inhibited the growth of fungal rhizospheres (Burke et al. 2014). Cerium oxide (CeO₂) NPs also did not influence the root elongation process of radish, rape, tomato, wheat, cabbage, and cucumber plants (Ma et al. 2010). SWCNTs labeled with FITC and DNA have no effect on the morphology, cytoplasmic fluidity, and cell survival of *Nicotiana tabacum* cells (Liu et al. 2009).

In addition to absorption, translocation of NPs from one plant part to another can spread toxicity and NPs can easily enter the food chain. Such NPs can thus undergo bioaccumulation followed by biomagnification. In such a study, Cerium (Ce) NPs

were absorbed by plant leaves but were not translocated from exposed leaves to fresh leaves or other plant parts (Birbaum et al. 2010). Accumulation of CeO₂ and ZnO NPs was observed in cucumber plants. NPs have been noticed to adhere on plant roots without blocking their water channels. However, both NPs did not affect their growth, gas exchange, and chlorophyll content (Zhao et al. 2013). ZnO NP exposure has also no effect on the overall vegetative growth of cowpea (*Vigna unguiculata*) (Wang et al. 2013a).

Exposure of aluminum (Al) NPs has no effect on the growth of California red kidney beans (*Phaseolus vulgaris*) and rye grass (*Lolium perenne*). However, aluminum accumulation was observed only in rye grass plants (Doshi et al. 2008). Pumpkin plants (*Cucurbita maxima*) also showed absorption, translocation, and accumulation of iron oxide (Fe₃O₄) NPs in roots and leaves from aqueous medium with no observed side effect on their growth and development (Zhu et al. 2008).

Stimulatory Effect of NPs on Seed Germination and Plant Vegetative Growth

Exposure of carbon nanotubes (CNTs), fullerenes, and graphene to rice seeds improved the seed germination rate and seedling growth compared to nontreated seedlings (Remya et al. 2012). Similarly, exposure of CNT has induced 200% increment in the fruit yield and vegetative growth of tomato (Husen and Siddiqi 2014). TiO₂ NP exposure has, likewise, documented improvement in the seed germination and growth of spinach plants (Zheng et al. 2005; Hong et al. 2005a; Lei et al. 2007; Su et al. 2007; Zheng et al. 2007; Su et al. 2008a; Xuming et al. 2008).

Influence of NPs on plant root growth has been reported as an important stress indicator. Root elongation is beneficial for plant growth and development, while decrease in root length negatively affects plant growth and hence indicates toxicity. Nonfunctionalized SWCNTs have been documented to induce root elongation in onion and cucumber plants (Canas et al. 2008). Root elongation was evident in *Brassica juncea* on MWCNT exposure. However, CNTs did not affect their seed germination (Gajanan et al. 2010). Likewise, MWCNTs were found to increase the seed germination and shoot–root length of *Onobrychis arenaria* (Smirnova et al. 2012). Oxidized MWCNTs were also noticed to promote the growth of wheat plants. The roots showed faster growth; however, no variation in seed germination rate and stem elongation was observed. Further, a considerable increment in plant vegetative biomass was also evident (Wang et al. 2012a). TiO₂ NP exposure has been documented to promote the process of root elongation in oilseed rape, kidney bean, and lettuce plants (Song et al. 2013).

Iron (Fe) NP exposure was reported to enhance the root length of *Arabidopsis thaliana* plants (Kim et al. 2014). Interestingly, graphene ribbons have been documented to increase the germination of 2-year-aged wheat seeds by 15%. Further, the developed plants showed better root differentiation and resistance to oxidative stress compared to untreated plants (Hu and Zhou 2014). CeO₂ NP exposure enhanced the root–shoot length and biomass accumulation in *Coriandrum sativum* plants. The

NPs also increased the enzymatic activity of antioxidant enzymes, catalase, and ascorbate peroxidase (Morales et al. 2013).

Polyhydroxyl fullerenes were documented to positively regulate the growth of *A. thaliana*, *Apergillus niger*, and *Ceriodaphnia dubia*. Increment in lifespan and reproduction efficiency of *C. dubia* and the vegetative growth parameters of *A. thaliana* like enhanced length of hypocotyls were noticed on fullerene exposure (Gao et al. 2011).

Interestingly, NP exposure has been reported to enhance the content of medicinally important phytochemicals. Carbon NPs and fullerols were also found to enhance the growth, metabolism, and yield of bitter melon plants. Exposure of fullerols to bitter melon seeds has increased their vegetative biomass and relative water content. Further, increment in the number of fruits, fruit length, and fruit weight enhanced their overall yield. Accumulation of anticancerous metabolites, that is, cucurbitacin-B and lycopene, was also significantly increased. Additionally, increment in the content of antidiabetic metabolites, charantin and insulin, was also evident (Kole et al. 2013).

PEG and carbon-coated silver (Ag) NPs were observed to stimulate the growth of *A. thaliana* and poplar plants. Large-sized Ag NPs were phytostimulatory whereas small-sized Ag NPs induced toxicity to plants. On the other hand, Ag⁺ ions were inducing stress and inhibiting their growth. Ag NPs of 25 nm were enhancing the root growth, biomass accumulation, and transpiration of both plants (Wang et al. 2013b). Application of root ash NPs to cucumber plants was found to enhance the shoot and root biomass (Moghaddasi et al. 2015). Foliar spray of gold (Au) NPs to *Brassica juncea* in field trials was found to enhance their vegetative growth. An increase in the number of leaves, branches, pods, and enhancement in their stem height and diameter was noticed. An overall increase in the yield of plants was also noticed (Arora et al. 2012). Similarly, exposure of Au NPs through Murashige and Skoog medium enhanced the vegetative growth and seed yield of *A. thaliana* plants (Kumar et al. 2013).

Exposure of CeO₂ NPs was found to reduce the seed germination and root–shoot length of radish seedlings. Modification of CeO₂ NPs with citric acid was found to enhance the root biomass and fresh water content of the treated seedlings (Trujillo-Reyes et al. 2013). Tetramethylammonium hydroxide-coated ferrophase magnetic NP exposure has growth-enhancing effect on maize plants (Racuciu and Creanga 2007). Citric acid-capped CeO₂ NPs increased the root biomass in radish (*Raphanus sativus*). Bare NPs, however, were toxic and induced reduction in root elongation and stem biomass (Trujillo-Reyes et al. 2013). CeO₂ NPs increased the biomass of kidney beans (Majumdar et al. 2014). TiO₂ NP treatment has induced plant growth enhancement in spinach. TiO₂ NP exposure has been reported to enhance the fresh weight, dry weight content, and overall growth of spinach plants (Gao et al. 2006; Yang et al. 2006; Linglan et al. 2008). TiO₂ NP exposure can overcome the nitrogen deficiency in spinach. Spinach plants showed nitrogen deficiency symptoms, chlorosis, when grown in N-deficient Hoagland solution. However, TiO₂ NP-treated spinach plants grown in nitrogen-deficient Hoagland solution showed normal growth compared to control plants (Yang et al. 2007).

MWCNTs induced growth of tobacco cells in cell culture medium (Khodakovskaya et al. 2012). Single-walled carbon nanohorns (SWCNHs) also enhanced the growth of tobacco cells in culture. SWCNHs also enhanced the seed germination and growth of barley, corn, rice, soybean, switchgrass, and tomato (Lahiani et al. 2015).

NP-Mediated Plant Tolerance to Stress and Disease

Xanthomonas perforans infection is known to reduce the fruit yield of tomato. Nanocomposite containing DNA-Ag NP-graphene oxides has been reported to maintain the growth and yield of *X. perforans*-infected tomato plants (Ocoy et al. 2013). In addition to the growth-promoting effect, TiO₂ NPs were also reported to act as antibacterial agents in potato tissue culture growth medium (Safavi 2014). The exposure of ultraviolet (UV)-B light has been known to induce oxidative damage in plant chloroplast. TiO₂ NPs were reported to prevent this oxidative damage in the chloroplast of spinach plants (Lei et al. 2008).

CNT has been reported to alleviate the stress induced by hyperaccumulation of cadmium (Cd) in *Spartina alterniflora* (Chai et al. 2013). Chlordane and DDX are nondegradable, persistent, and estrogenic pollutants. Exposure of MWCNTs inhibited the accumulation of chlordane and DDX in corn, soybean, tomato, and zucchini plants. A higher dose of MWCNTs was required to inhibit the pollutant uptake in soybean than corn, tomato, and zucchini plants. Similarly, differential response of plant toward pesticide accumulation was noticed in the presence of C₆₀ fullerenes. A variable and inconsistent reduction in the accumulation of chlordane and DDX was observed in zucchini and corn plants on exposure to C₆₀ fullerenes. However, for soybean and tomato, the process of pesticide uptake was independent of C₆₀ presence (Torre-Roche et al. 2013). Likewise, TiO₂ NPs has also been reported to compensate the phytotoxic chemical, linolenic acid that negatively regulates the process of photosynthesis (Su et al. 2008b).

10.2.2 Plant–NP Interaction: Negative Effects

The negative effect of NPs on plant growth and development indicates their phytotoxic nature (Fig. 10.4). Exposure of NPs can lead to phytotoxicity either at all tested doses or above a threshold dose. Several reports have documented the detrimental effects of NPs on plants.

Negative Effect on Seed Germination and Root and Shoot Elongation

Roots are the foremost and prime plant tissue that comes in contact with the NPs on exposure. NPs are initially accumulated in the roots and later transported to various plant tissues. This process of uptake and transport of NPs can induce several morphological, anatomical, and physiological plant responses.

As mentioned earlier, reduction in plant root length on NP exposure demonstrates their phytotoxicity on plants. Exposure of alumina NPs has been documented to inhibit the root elongation in maize, *Cucumis sativus*, *Glycine max*, *Brassica oleracea*, and *Daucus carota* (Yang and Watts 2005). Nonfunctionalized and poly-3-aminobenzenesulfonic acid-functionalized SWCNTs inhibited the elongation of tomato and lettuce roots, respectively (Canas et al. 2008). Yb₂O₃, Gd₂O₃, and La₂O₃ NP exposure reportedly inhibited the root elongation of lettuce, rape, and wheat plants. However, CeO₂ NPs hampered the growth of only lettuce roots (Ma et al. 2010). Palladium (Pd), copper (Cu), Si, and Au NPs have also been documented for root growth retardation in lettuce (Shah and Belozerova 2009). Lanthanum oxide (La₂O₃) NPs were also reported to induce toxicity to cucumber plants (Ma et al. 2011). Au NPs has also induced leaf necrosis in tobacco plants (Sabo-Attwood et al. 2012). In another study, the seed germination and root elongation of ryegrass and corn was inhibited in the presence of Zn and ZnO NPs (Lin and Xing 2007). SWCNTs were found to inhibit the growth of root hair in maize (Yan et al. 2013). CdS QDs were observed to inhibit the growth and physiology of wild and Ds element expressing mutant *A. thaliana* plants (Marmioli et al. 2014).

Fullerene exposure was observed to reduce hypocotyl length and root length of germinated *A. thaliana* seedlings. Further, root gravitropism responses of exposed roots were also inhibited (Liu et al. 2010). Copper oxide (CuO) exposure has reduced the root length followed by reduced shoot length of *P. vulgaris* (Dimkpa et al. 2015). ZnO NP exposure also induced inhibition of seed germination and growth in rapeseed (*Brassica napus*). ZnO NPs inhibited the root–shoot elongation and dry weight of the exposed shoots (Kouhi et al. 2014).

Exposure of Ag NPs was found to reduce the germination and growth of rice seedlings. Further, the shoot–root growth of NP-exposed seedlings was considerably inhibited (Thuesombat et al. 2014). In a comparative toxicity study, polyvinyl pyrrolidone- and gum arabic-coated Ag NPs inhibited seed germination and vegetative growth of *Phytolacca americana* (Yin et al. 2012). Ce-coated Ag NPs were observed to inhibit the growth of *P. radiatus* and *Sorghum bicolor* in agar and soil medium. The phytotoxic responses were lowered when NPs were exposed through soil (Lee et al. 2012). Cu NPs inhibited the growth of *P. radiatus* and *Triticum aestivum* plants. However, growth inhibition was more pronounced in *P. radiatus* than in *T. aestivum* (Lee et al. 2008).

Ag NPs inhibited seedling growth and development of root hairs in *Lolium multiflorum* (Yin et al. 2011). Fullerenes have inhibited the vegetative growth of tobacco (Liu et al. 2013). Exposure of Ag NPs was found to prolong the vegetative growth of *A. thaliana* plants by 2–3 days, while their reproductive growth was shortened by 3–4 days as compared to untreated plants. The seed germination of treated plants

was drastically decreased for the next three generations. However, no morphological alteration in the NP-exposed plants was evident (Geisler-Lee et al. 2014).

MWCNTs have induced reduction in shoot and root length of red spinach, lettuce, rice, and cucumber plants. Further, cell death and electrolyte leakage were also noticed in these plants (Begum et al. 2012). MWCNTs were also reported to reduce seed germination, seedling growth, and development of barley, corn, and soybean (Lahiani et al. 2013). CeO₂ and TiO₂ did not affect the seed germination but reduced root elongation in *Hordeum vulgare* L (Mattiello et al. 2015). Ag NPs have inhibited the overall vegetative growth of *Crambe abyssinica* (Ma et al. 2015). CuO NPs also inhibited the growth of the maize plant. The NPs, however, have no effect on seed germination (Wang et al. 2012b). Citrate-capped Ag NPs inhibited maize seed germination. ZnO NPs did not affect the germination of maize. Both ZnO and Ag NPs induced deformation of root cells and irregular growth of maize roots (Pokhrel and Dubey 2013). Ag NPs inhibited the *Triticum aestivum* L. seedling growth. NPs also altered the morphology of root tip cells (Vannini et al. 2014).

Negative Effect of NPs on Plant Biomass and Chlorophyll Content

There are several reports documenting the influence of NP exposure on biomass, vegetative growth, and physiological aspects, such as chlorophyll accumulations of plants. Exposure of MWCNTs and C₇₀ fullerenes in combination with natural organic matter was reported to retard plant growth and extend the flowering time and maturity age of rice seeds (Lin et al. 2009a). ZnO NPs has induced reduction in *Lolium perenne* (ryegrass) biomass. Further, the NPs induced shrinkage of root tips and collapse of root epidermal and cortical cells (Lin and Xing 2008). ZnO NPs induced reduction in biomass weight and induced damage to *Fagopyrum esculentum* roots (Lee et al. 2013). *Cucurbita pepo* (zucchini) plants exposed to hydroponic suspension of Ag NPs have also shown growth retardation. Exposure of Cu NPs has induced reduction in root length and plant biomass accumulation. While Ag NP exposure decreased the transpiration rate in addition to reduction in plant biomass (Stampoulis et al. 2009). Exposure of variously sized MWCNT agglomerates showed size-dependent deteriorating effect on dry weight, viability, and chlorophyll content of T87 *A. thaliana* suspension cells. MWCNTs also lowered the activity of superoxide dismutase enzyme of these cell lines in a size-dependent manner (Lin et al. 2009b). Perchloric acid-coated iron oxide NPs have reduced the chlorophyll a/chlorophyll b ratio of maize plants. NP exposure also reduced the stem length. These variations along with stimulation of nucleic acid biosynthesis were more pronounced in the presence of electromagnetic field (Racuciu et al. 2009). Exposure of C₆₀ fullerenes was found to reduce the growth, chlorophyll content, and chloroplast oxygen production of aquatic plant, *Lemna gibba* (Santos et al. 2013). C₆₀ fullerenes are thus documented as a threat to the aquatic ecosystem.

TiO₂ NPs were found to reduce the fresh and dry weight contents of wheat shoots and roots. Further, reduction in the chlorophyll content of exposed plants was noticed (Mahmoodzadeh et al. 2013). Tomato plants exposed to Ag, Co, Ni, CeO₂, Fe₃O₄, SnO₂, and TiO₂ NPs also showed reduction in stem length. Additionally,

reduction in biomass of lower leaf, stem, and root was noticed (Antisari et al. 2015). Likewise, Ag NPs retarded the growth of *Brassica campestris* and *Vigna radiata* plants. Further, transmission electron microscopy evidenced the breakage of the cell wall on Ag NP exposure (Mazumdar 2014). A comparative study of Ag⁺ ion and Ag NP exposure revealed more toxic effects of NPs on *A. thaliana* as compared to Ag⁺ ions. Ag NP exposure decreased the root length, chlorophyll content, and water homeostasis of *A. thaliana* plants. Ag NPs also disturbed the thylakoid structure of chloroplasts and reduced the transcript expression of antioxidant and aquaporin genes (Qian et al. 2013). CeO₂ NPs induced oxidative stress and growth inhibition in *A. thaliana*. The chlorophyll content of CeO₂ NP-exposed plants was also reduced. Exposure of In₂O₃ NPs did not alter the biomass, total chlorophyll, and antioxidant potential of *A. thaliana* plants. Only minor root growth inhibition was observed on exposure of In₂O₃ NPs (Ma et al. 2013). Si NPs induced chlorosis and reduction in rosette diameter, biomass, and stem length in *A. thaliana* (Slomberg and Schoenfisch 2012).

NP-Induced Oxidative Stress on Plants

The antioxidant potential of plants protects them from internal and external oxidative stress. If NPs disturb the antioxidant potential of plants, their growth and development will be directly or indirectly affected. A few studies reported negative effect on plant growth mainly due to oxidative stress induced by NPs. In such a study, graphene exposure induced oxidative stress and inhibited the germination, growth, and biomass accumulation of cabbage, red spinach, and tomato plants. Interestingly, graphene showed no ill effect on the growth and development of lettuce plants (Begum et al. 2011). Exposure of MWCNTs increased the generation of reactive oxygen species (ROS) in spinach plants. MWCNT-treated plants showed cell damage and alteration in leaf and root anatomy. However, supplementing the plant growth medium with ascorbic acid, an antioxidant compound was found to alleviate the oxidative stress induced by MWCNTs (Begum and Fugetsu 2012). Likewise, graphene oxide sheets induced oxidative stress in *V. faba* plants (Anjum et al. 2013). CeO₂ exposure induced oxidative stress and has negative effect on growth of rice seedlings (Rico et al. 2013a). CeO₂ NP exposure also induced the formation of ROS. NPs also inhibited the germination and root growth of lettuce (Cui et al. 2014). Graphene exposure was reported to induce apoptosis in T87 *A. thaliana* cell lines (Begum and Fugetsu 2013). ZnO NPs have no effect on the shoot growth, although oxidative stress was induced in leaves of green pea (*Pisum sativum*). However, root elongation was induced on ZnO NP exposure (Mukherjee et al. 2014).

Deleterious Effects of NPs on the Genetic Constitution of Plants: Genotoxicity

Exposure of Ag NPs induced alteration in cell division and genotoxicity to *Allium cepa* roots (Kumari et al. 2009). Likewise, magnetic NP exposure has also been documented to influence the root cell growth and development of maize plants (Racuciu and Creanga 2009a). Exposure of SWCNTs was found to induce programmed cell death in *A. thaliana* and rice plants (Shen et al. 2010). *A. thaliana* plants showed disrupted root growth on TiO₂ NP exposure (Wang et al. 2011). TiO₂ NPs were also observed to negatively affect the seed germination and root elongation of *Vicia narbonensis* and maize plants (Castiglione et al. 2011). TiO₂ NPs also inhibited the germination and growth of *Vicia narbonensis* (Castiglione et al. 2014). Ag NPs have also been reported to lower the reproduction efficiency of kiwi plants (Speranza et al. 2013). So, NPs have the potential to disturb the genetic makeup of plants, which ultimately affect their growth, development, and physiology. Further, being genetic defects, these ill effects of NP exposure can be transferred to coming generations.

Negative Effect of NPs on Plant Nutritional Quality/Status

Exposure of CeO₂ NPs was found to reduce the nutritional quality of rice seedlings. The seedlings showed reduction in iron, sulfur, prolamine, glutelin, lauric acid, valeric acid, and starch content. Further, the antioxidant content of seedlings was also reduced. Thus, CeO₂ exposure has a deteriorating effect on the nutritional value of rice (Rico et al. 2013b). CeO₂ NPs also reduced the fresh weight of fruits in cucumber plants (Zhao et al. 2014). Similarly, ZnO NPs decreased the iron content and increased the zinc accumulation of soybean plants. However, CeO₂ exposure decreased the uptake of elements involved in photosynthesis and nitrogen metabolism of soybean (Peralta-Videa et al. 2014). Ag NPs were reported to induce mild stress on aquatic plant, *Bacopa monnieri* (Krishnaraj et al. 2012). Ag NPs and FeO NPs were observed to disturb the nutrient uptake and the growth of the mycorrhizal clover (*Trifolium repens*) plant. The lower dose of Ag NPs induced more toxicity than a higher tested dose. In contrast, a higher dose of FeO NPs induced more plant growth inhibition than a lower dose (Feng et al. 2013). MWCNTs also induced nutrient imbalance and damage to leaves in *Vicia faba* (Wang et al. 2014).

10.3 Mechanism Regulating Plant–NP Interactions

Exposure of different type of NPs can variously affect plant growth and development. The NPs of even the same chemical composition can positively as well as negatively affect the plants depending upon their size, shape, surface covering, concentration, and mode of NP exposure. Hence, it becomes essential to investigate the mechanism of NP interaction with plants.

10.3.1 Mechanism Underlying the Positive Effect of NPs on Plants

Exposure of some type of NPs can have positive effect on the growth of plants as discussed (Table 10.1). Some studies have even documented the probable mechanism underlying the growth-promoting abilities of NPs.

Table 10.1 Positive effects of different types of NPs on plants

Sr. N.	Type of NPs	Response	References
1.	Ag NPs	Enhanced root growth, biomass accumulation and evapo-transpiration; protected plants from heavy metal stress and AMF infection	Feng et al. (2013), Wang et al. (2013b)
2.	Au NPs	Enhanced carbohydrate content and antioxidant potential and overall yield	Arora et al. (2012)
3.	Carbon NPs and fullerols	Enhanced relative water content, growth, metabolism, and accumulation of anticancerous and antidiabetic metabolites; increment in the number of fruits, fruit length, and fruit weight	Kole et al. (2013)
4.	CeO ₂	Enhanced biomass accumulation, root–shoot length, fresh water content, macro- and micronutrient absorption, activity of antioxidant enzymes, catalase, and ascorbate peroxidase	Morales et al. (2013), Trujillo-Reyes et al. (2013), Majumdar et al. (2014)
5.	CNT	Enhanced biomass accumulation, vegetative growth and fruit yield; alleviated stress induced by hyperaccumulation of cadmium	Khodakovskaya et al. (2012), Chai et al. (2013), Husen and Siddiqi (2014)
6.	C ₆₀ fullrenes	Enhanced seed germination, water retention, seedling growth, and phytochemical accumulation; alleviated stress induced by exogenous pesticide	Remya et al. (2012), Torre-Roche et al. (2013), Husen and Siddiqi (2014)
7.	DNA-Ag NP-graphene oxide nanocomposite	Enhanced plant growth and resistance to <i>X. perforans</i>	Ocsoy et al. (2013)
8.	Fe	Enhanced root length	Kim et al. (2014)
9.	Ferrophase magnetic NPs	Enhanced chlorophyll content, nucleic acid levels, and photosynthesis rate	Racuciu and Creanga (2007)
10.	Graphene ribbons (HGR)	Enhanced germination, better root differentiation, and resistance against oxidative stress	Hu and Zhou (2014)

(continued)

Table 10.1 (continued)

Sr. N.	Type of NPs	Response	References
11.	MWCNT	Enhanced seed germination and shoot–root growth	Gajanan et al. (2010), Smirnova et al. (2012), Wang et al. (2012a)
12.	PHF	Enhanced hypocotyl length and vegetative growth; increment in lifespan and reproduction efficiency	Gao et al. (2011)
13.	Root ash NPs (RANPs)	Enhanced shoot–root biomass and zinc accumulation	Moghaddasi et al. (2015))
14.	SWCNT and SWCNH	Enhanced seed germination and root elongation	Canas et al. (2008), Lahiani et al. (2015)
15.	TiO ₂	Enhanced seed germination, root–shoot elongation fruit yield, photosynthetic rate of plants, Increased chlorophyll content, carbon assimilation, ribulose biphosphate carboxylase/oxygenase activity, nitrogen absorption and assimilating; protection against oxidative damage of chloroplast, bacterial agents, and phytotoxic chemicals such as linolenic acid	Zheng et al. (2005), Gao et al. (2006), Yang et al. (2006), Yang et al. (2007), Lei et al. (2008), Linglan et al. (2008), Su et al. (2008a), Song et al. (2013), Safavi (2014), Qi et al. (2013)

NPs Enhance Water Uptake

Various reports document enhanced seed germination on NP exposure. CNTs, fullerenes, and graphene improved the germination of rice seed by increasing their water intake. Due to better water content, the nanomaterial-treated seedlings were healthier than the untreated seedlings (Remya et al. 2012). Au NPs were also reported to improve the water and oxygen permeability of seed capsules that improved seed germination in *B. juncea*. Further, increase in light absorption, carbohydrate content, increased gibberellin, and total chlorophyll content was responsible for the enhanced vegetative growth of the plants. Au NP exposure also improved the antioxidant potential of the treated plants (Arora et al. 2012). Fullerenes increased the water uptake of bitter melon seeds and enhanced their overall productivity in terms of number of fruits (Kole et al. 2013). Fullerenes and CNT treatment increased the water retention ability of tomato plants. As a result of more water retention, the treated plants accumulated more cucurbitacin, lycopene, and insulin. Better water retention was responsible for enhanced vegetative growth and fruit production of tomato (Husen and Siddiqi 2014).

NPs Upregulated Photosynthesis and Secondary Metabolism

TiO₂ NPs enhanced the vegetative growth of spinach plants by increasing their photosynthetic efficiency and photosynthetic rate. TiO₂ NPs were found to increase the chlorophyll content, ribulose biphosphate carboxylase/oxygenase activity, and photosynthetic rate of plants (Zheng et al. 2005). TiO₂ NPs reportedly increased the absorption of light in the red and blue region of spinach. Enhancement in the excitation energy absorbed by light harvesting complex (LHC) II was observed, which further increased the energy transferred from photosystem PS I to PS II (Su et al. 2007). TiO₂ NPs also increased the LHC II content in the thylakoid membranes of spinach plants. NPs entered the spinach chloroplasts and interacted with PS II to promote the transfer of energy from chlorophyll b and carotenoids to chlorophyll a. Hence, the process involving the conversion of light energy to electron energy namely, electron transport, water photolysis, and oxygen evolution were also accelerated (Zheng et al. 2007). Later, TiO₂ NPs were also reported to improve ATP synthesis and transport across the membrane. Enhanced chloroplast coupling and increased activity of the ATP synthase enzyme were responsible for enhanced growth of plants on NP exposure. The noncyclic photophosphorylation activity of chloroplasts was higher than cyclic photophosphorylation activity on NP exposure. Simultaneously, easier entry of Ca²⁺ and Cl⁻ was also favored into the oxygen-evolving complex during NP exposure (Hong et al. 2005a; Lei et al. 2007). TiO₂ NPs have also been reported to promote electron transport by accelerating the primary charge separation of PS II and P680⁺ (Su et al. 2008a). This was further supported by the fact that TiO₂ NPs also enhanced the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) enzyme. They increased the protein expression and activity of RUBISCO enzyme (Gao et al. 2006; Linglan et al. 2008). Hence, these factors were collectively responsible for the better growth of spinach on TiO₂ NP exposure.

Nitrogen is the main component required for the normal structure and function of chlorophyll. TiO₂ NP exposure was found to increase the nitrogen assimilation of spinach plants. These NPs accelerated the direct reduction of NO₃⁻ to NH₄⁺. Subsequently, there was enhanced transformation of NH₄⁺ into organic nitrogen like proteins and chlorophyll. Simultaneously, the activities of important nitrogen-assimilating enzymes were also enhanced on NP exposure (Yang et al. 2006). Ag NPs induced the blockage of ethylene receptors and enhanced indole-3-acetic acid efflux from plant roots to induce phytostimulatory effects on *A. thaliana* and poplar plants (Wang et al. 2013b). Graphene ribbons improved the vegetative growth and metabolism of wheat by improving their secondary metabolism and nitrogen sequestration. Graphene ribbon exposure was found to enhance the accumulation of carbohydrates, amino acids, and fatty acids. Ribbons also improved the integrity and permeability of cell membranes (Hu and Zhou 2014). TiO₂ NPs were also reported to increase the photosynthesis in tomato leaves under mild heat stress conditions. TiO₂ NPs induced increase in the transpiration rate and regulated photosystem II (PS II) energy dissipation. The nonregulated PS II energy dissipation was

decreased. These factors collectively resulted in overall increase in the rate of photosynthesis on TiO₂ NP exposure under heat stress (Qi et al. 2013).

NPs Change Genetic Material Expression

TiO₂ NPs increased the messenger RNA (mRNA) expression of both small (*rbcS*) and large (*rbcL*) subunits of RUBISCO in spinach. The protein expression and enzyme activity of RUBISCO was 2.33 times higher in TiO₂ NP-treated plants than control plants (Xuming et al. 2008). While Au NPs were found to enhance the seed yield and vegetative growth of *A. thaliana* by inducing the key plant-regulating molecules, microRNAs. Exposure of Au NPs induced alteration in the expression of microRNAs related to plant growth and development (Kumar et al. 2013). SWCNHs regulated the expression of stress responses, cellular responses, and metabolic processes to enhance the seed germination and growth of various crop plants (Lahiani et al. 2015). The tobacco cell growth-enhancing effect of MWCNTs was due to the upregulation of *CycB*, *NtLRX1*, and aquaporin genes. Unregulated genes increased the water transport and cell division of treated cells as compared to control tobacco cells (Khodakovskaya et al. 2012).

NPs Mediated Increase in Nutrient Absorption

Citric acid-coated CeO₂ NPs have enhanced the growth parameters of radish seedlings by increasing the macro- and micronutrient absorption of plants. Coated NPs also increased the water uptake of roots and resulted in their increased biomass. Noncoated CeO₂ NPs, however, aggregated in the medium and induced toxicity (Trujillo-Reyes et al. 2013). Tire rubber ash NPs release zinc and iron that increased their availability to cucumber. As zinc and iron are important for growth and development of plants, NP exposure was indirectly enhancing the root–shoot growth of cucumber plants (Moghaddasi et al. 2015).

NPs Changed Cell Architect

CeO₂ NPs were observed in the form of cerium perhydroxide in the cell wall and intercellular spaces of epidermal and cortical cells of *C. sativum* plants. However, their presence was not indicated in the meristematic tissues. Hence, decreased enzymatic stress in the growing zone was responsible for the enhanced growth of plants on CeO₂ exposure (Morales et al. 2013). Exposure of MWCNTs induced cellular elongation of root cells and increased the activity of dehydrogenase enzyme of wheat plants (Wang et al. 2012a). Exposure of Fe NPs was reported to induce root elongation by free radical-mediated cell wall loosening of *A. thaliana* roots. NPs induced cell wall loosening by generation of H₂O₂ that released OH free radicals and degraded the pectin-polysaccharides of plant roots. NPs also induced more

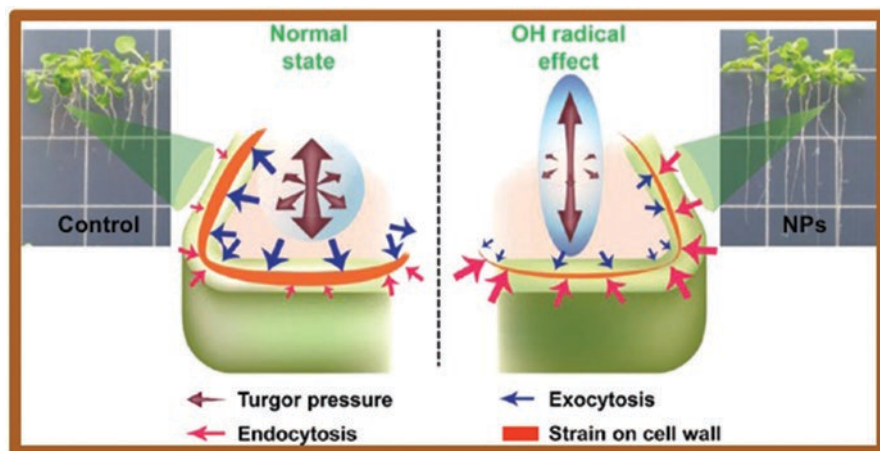


Fig. 10.5 Image showing Fe NP-induced hydroxyl radical-induced cell wall loosening due to enhanced endocytosis and reduced exocytosis in root cells of *A. thaliana*. However, in control plant roots, reverse order of endocytosis and exocytosis was observed. Hence, NPs induced longitudinal turgor pressure leading to root elongation. “Reprinted with permission from (Kim et al. 2014). Copyright (2014) American Chemical Society”

longitudinal turgor pressure that led to root elongation as depicted in Fig. 10.5 (Kim et al. 2014). So these changes in cell architecture were collectively responsible for the increased root length and biomass of plants.

NPs Enhanced Tolerance to Plant Stress and Disease

Nitrogen deficiency has no effect on the growth of TiO_2 NP-treated spinach. TiO_2 NP treatment has induced reduction of N_2 to NH_3 in spinach grown in nitrogen-deficient Hoagland solution. The organic nitrogen produced by NH_3 transformation was used for chlorophyll or protein biosynthesis to overcome nitrogen deficiency (Yang et al. 2007). Hence, plants can survive nitrogen deficiency if supplemented with TiO_2 NPs. TiO_2 NPs not only increased the photosynthetic performance of spinach plants, but it also alleviated the electron transport inhibitory activity of linolenic acid. Exposure of TiO_2 NPs accelerated the overall electron transport. This compensated for the damage caused by linolenic acid to the structure and function of chloroplast in spinach (Su et al. 2008b). TiO_2 NPs also restored the rigidity and hydrophobicity of membranes destroyed by linolenic acid. The positive charge of TiO_2 NPs has neutralized the negative charge of lipid-protein present on the membranes. As a result, repulsion between thylakoid membranes was reduced which favored the stacking of thylakoid membranes. Similarly, TiO_2 NPs also prevented and repaired the UV-B radiations induced oxidative damage of chloroplasts membrane. TiO_2 NPs absorbed the UV-B radiations, thereby reducing the exposure to chloroplast. Simultaneously, NPs enhanced the activities of antioxidant enzymes,

catalase, and glutathione peroxidase (Hong et al. 2005b; Lei et al. 2008). Hence, TiO₂ NPs could maintain plant growth in prevailing stress conditions.

Likewise, CNTs were reported to alleviate the Cd stress by regulating the accumulation of various cations and organic solutes. CNT counterbalanced the cadmium stress by enhancing the K⁺ and Ca⁺ concentrations and reducing the levels of proline and soluble carbohydrates (Chai et al. 2013). Similarly, MWCNTs and C₆₀ fullerenes reduced the pesticide absorption of zucchini, corn, tomato, and soybean plants. These nanostructures have high affinity for hydrophobic pesticides, chlordane and DDX, and thus prevent their uptake by plants (Torre-Roche et al. 2013). The nanocomposite Ag@dsDNA@GO was found to impart bacterial resistance to tomato plants. GO nonspecifically interacted with the bacteria and wrapped it by inducing deformation in its rod-shaped structure. While Ag induced cell membrane destruction and provided bacterial tolerance to plants. Nanocomposites were found to reduce the viability of *X. perforans* in culture as well as on plants. Thus, nanocomposites maintained the tomato growth and yield in *X. perforans*-infected tomato (Ocsoy et al. 2013).

10.3.2 Mechanism Underlying the Negative Effects of NPs on Plants

Various reports have documented the growth-inhibitory effect of NPs on plants (Table 10.2). The mechanism underlying the phytotoxic nature of NPs is discussed as under.

NPs Reduced Chlorophyll Content and Inhibited Photosynthesis

Ferrophase magnetic NPs were inhibiting the growth of maize plants by influencing their photosynthetic reactions. NPs penetrated the nuclear biomembrane and interfered with the nucleic acid biosynthesis. The magnetic properties of NPs also influenced the transmembrane ion flow and affected the structure of enzymes involved in photosynthesis. NPs also altered the metabolism at various tissue levels (Racuciu and Creanga 2007, 2009b). Likewise, the exposure of Ag NPs altered the biochemical composition of *B. monneri* to induce stress. Exposure of Ag NPs decreased the total phenolic, protein, and carbohydrate content of plants. NPs also reduced the level of proteins associated with the photosystem, starch synthesis system, and carbohydrate translocation machinery (Krishnaraj et al. 2012). The response of NPs on plants can further vary depending upon the environmental and exposure conditions. The growth inhibitory effects of TiO₂, CeO₂, and Cu(OH)₂ NPs to *Clarkia unguiculata* were noticed at some specific conditions. Exposure of TiO₂ and CeO₂ at maximum plant growth period, high light, and nutrient conditions disrupted the energy transfer from photosystem II (PSII) to the Calvin cycle. As a result, the

Table 10.2 Negative effects of NPs on plants

Sr. No.	Type of NPs	Response	References
1.	Ag NPs	Inhibited seed germination, shoot–root growth, transpiration, biomass accumulation, and transcript expression of antioxidant and aquaporin genes; decreased chlorophyll content; disturbed water homeostasis, chromosomal structure, and thylakoid structure of chloroplasts; reproductive growth of plants shortened by 3–4 days; trophic transfer	Kumari et al. (2009), Stampoulis et al. (2009), Yin et al. (2011), Lee et al. (2012), Yin et al. (2012), Pokhrel and Dubey (2013), Qian et al. (2013), Mazumdar (2014), Thuesombat et al. (2014), Vannini et al. (2014), Ma et al. (2015), Yasur and Pathipati (2015)
2.	Al NPs	Inhibit root elongation	Yang and Watts (2005)
3.	Au NPs	Leaf necrosis; trophic transfer	Judy et al. (2011), Sabo-Attwood et al. (2012), Judy et al. (2012), Unrine et al. (2012)
4.	QDs	Inhibited growth and physiological metabolism; trophic transfer	Marmiroli et al. (2014), Koo et al. (2015)
5.	CeO ₂	Inhibited root–shoot growth; generated free radicals, H ₂ O ₂ ; reduced antioxidant potential, chlorophyll content, lignin content and activities of antioxidant enzymes; induced oxidative stress, enhanced membrane damage, electrolyte leakage; nutritionally deficient seed formation	Ma et al. (2013), Rico et al. (Rico et al. 2013a, b), Conway et al. (2015), Du et al. (2015), Majumdar et al. (2015), Mattiello et al. (2015), Ma et al. (2016), Majumdar et al. (2016)
6.	Co, Ni, CeO ₂ , Fe ₃ O ₄ , SnO ₂ and TiO ₂ NPs	Inhibited stem length, biomass accumulation of lower leaf, stem, and root; trophic transfer	Mahmoodzadeh et al. (2013), Antisari et al. (2015)
7.	Cu, CuO and Cu(OH) ₂	Inhibited root length, shoot length, and plant biomass accumulation	Lee et al. (2008), Stampoulis et al. (2009), Wang et al. (2012b), Conway et al. (2015), Dimkpa et al. (2015)
8.	Fe oxide and magnetic NPs	Inhibited stem length; reduced chlorophyll a/b ratio; induced chromosomal aberrations	Racuciu et al. (2009), Racuciu and Creanga (2009a)
9.	Fullrenes	Inhibited hypocotyl growth, root elongation; disturbed root gravitropism responses; disruption of cell wall and cell membrane of roots	Liu et al. (Liu et al. 2010, Liu et al. 2013)
10.	Graphene oxide sheets	Induced oxidative stress to plants	Anjum et al. (2013)

(continued)

Table 10.2 (continued)

Sr. No.	Type of NPs	Response	References
11.	MWCNTs and C ₆₀ NPs	Inhibited seed germination, biomass accumulation, activity of SOD enzyme; reduced shoot–root length and chlorophyll content; induced generation of ROS, cell death, electrolyte leakage, cellular damage, and alteration in leaf and root anatomy; delayed flowering time and maturity age of plants	Lin et al. (2009a, b), Stampoulis et al. (2009), Begum and Fugetsu (2012), Begum et al. (2012) Lahiani et al. (2013), Santos et al. (2013), Wang et al. (2014)
12.	Pd, Cu, Si NPs	Inhibited biomass accumulation, leaf, root and stem growth; induced chlorosis in leaves	Padmadhas and Ragunathan (2009), Shah and Belozeroova (2009), Slomberg and Schoenfish (2012)
13.	SWCNT	Inhibited root hair growth and root length; genes associated with root hair growth also downregulated; induced programmed cell death, cellular aggregation, chromatin condensation, plasma membrane deposition, and H ₂ O ₂ generation	Canas et al. (2008), Shen et al. (2010), Yan et al. (2013)
14.	TiO ₂	Inhibited seed germination and root elongation; disrupted microtubular network and mitotic index	Castiglione et al. (2011), Wang et al. (2011), Mattiello et al. (2015), Conway et al. (2015), Kubo-Irie et al. (2016)
15.	Yb ₂ O ₃ , Gd ₂ O ₃ and La ₂ O ₃ NPs	Inhibited root elongation and plant biomass accumulation; strophic transfer	Ma et al. (Ma et al. 2010, Ma et al. 2013), Roche et al. (2015)
16.	ZnO	Inhibited seed germination, biomass accumulation, root and shoot elongation; induced root tip shrinkage and collapse of root epidermal and cortical cells	Lin and Xing (Lin and Xing 2007, Lin and Xing 2008), Lee et al. (2013), Pokhrel and Dubey (2013), Geisler-Lee et al. (2014), Kouhi et al. (2014), Mukherjee et al. (2014)

photosynthetic rate of plants was decreased. In contrast, Cu(OH)₂ NPs lowered the rate of photosynthesis at unfavorable conditions, that is, high light, limited nutrient conditions (Conway et al. 2015). CuO NPs induced chlorosis that ultimately inhibited the growth of maize plants (Wang et al. 2012b).

NPs Altered Plant Maturity and Genetic Constitution

MWCNTs and fullrene C₇₀ were documented to delay flowering time and maturity age of rice plants. NPs entered the plant root through cell wall pores and intercellular plasmodesmata. Osmotic and capillary forces were responsible for NPs' entry into the roots. NPs aggregated inside the vascular tissues, thus halting the nutrient and water uptake by plants. The ill effects were more prominent in plants exposed

to C₇₀ NPs than MWCNTs (Lin et al. 2009a). SWCNTs were found to alter the expression of genes associated with seminal root and root hair growth of maize. SWCNTs induced upregulation of genes encoding epigenetic modification enzymes, causing deacetylation of H3 histones that affected root growth (Yan et al. 2013).

Magnetic NP exposure has increased the mitotic index and induced low percentage heritable chromosomal aberrations to maize plants (Racuciu and Creanga 2009a). Ag NPs have been reported to interact with pollen membranes and reduce their viability in kiwi. These NPs were more potent at disrupting the pollen tube elongation process by inducing ultrastructural alterations and changing their calcium content (pollen tube elongation is a critical step of fertilization in flowering plants). Exposure of PVP-coated Ag NPs induced the disruption of pollen tube elongation in kiwi fruits. This disruption has lowered their ability to reproduce (Speranza et al. 2013). Perchloric acid-coated Fe oxide NPs were documented to stimulate nucleic acid biosynthesis and reduce chlorophyll a/ chlorophyll b ratio and plant length in maize. The magnetic NPs were internalized by vegetal tissue and they absorbed electromagnetic energy. This induced putative local heating, leading to the observed metabolic process-mediated regeneration reactions (Racuciu et al. 2009). TiO₂ NPs were, likewise, found to reduce the mitotic index of *V. narbonensis* and maize plants. Disturbances induced in spindle apparatus, DNA strand breakage, and chromosomal aberrations were responsible for the reduction of plant mitotic index (Castiglione et al. 2011).

Ag NPs have induced genotoxic effects as a result of bridging of chromatin, disturbed metaphase, and multiple chromosomal breaks in *A. cepa* root cells (Kumari et al. 2009). CdS QD-treated *A. thaliana* showed the upregulation of 195 and downregulation of 43 genes. Among these genes, 32% of genes were involved in stress tolerance. Transgenic *A. thaliana* containing Ds elements confirmed that the Ds insertion site was responsible for countering QD toxicity (Marmioli et al. 2014).

TiO₂ NP exposure was inducing DNA damage in *V. narbonensis* plants. TiO₂ NPs did not induce H₂O₂ and ROS production, so ROS-independent DNA fragmentation mechanism was involved in TiO₂-mediated genotoxic effects. TiO₂ was found to interact directly with the phosphate groups of DNA and inducing damage (Castiglione et al. 2014). Graphene induced an increase in the expression of genes encoding water channel proteins in barley, corn, and soybean plants. These genes have induced more uptake of graphene through the water channels and hence enhanced the phytotoxic effects (Lahiani et al. 2013).

NPs Negatively Altered Plant Growth by Inducing Oxidative/Abiotic Stress

SWCNTs have induced oxidative stress in the leaves and protoplast of *A. thaliana* and rice cells. Oxidative stress was responsible for the initiation of programmed cell death in *A. thaliana* and rice plants (Shen et al. 2010). TiO₂ NPs have been reported to induce disorganization of microtubular and 26S proteasome-mediated tubulin degradation in *A. thaliana* root cells. ROS generation and physical interaction with

protein moieties of tubules on NP exposure were responsible for observed phytotoxicity (Wang et al. 2011). Graphene phytotoxicity has been identified in tomato, cabbage, and red spinach. Graphene induced the formation of a long primary root and several smaller lateral roots. This enhanced the further absorption of graphene. Graphene enhanced the production of H_2O_2 and thus induced cell death (Begum et al. 2011). Exposure of Ag NPs has led to their accumulation in roots and leaves of *A. thaliana*. Ag NP accumulation has induced accumulation of anthocyanin in plants. Anthocyanin accumulation increased the ROS generation in plants and hence, oxidative stress. ROS generation and the physical presence of Ag NPs were collectively responsible for the change in chlorophyll structure, reduced antioxidant potential, and downregulation of aquaporins (Qian et al. 2013). MWCNTs have also been reported to induce ROS-mediated oxidative stress in red spinach. MWCNTs have changed the morphology and internal structure of spinach roots. This alteration in root architect has enhanced the MWCNT uptake. This has further magnified the toxicity responses in red spinach (Begum and Fugetsu 2012). C_{60} NPs have induced ROS-mediated dysfunctioning of PS II in *L. gibba*. C_{60} NPs impaired the chloroplast functionality that disturbed the electron transfer between photosynthetic electron transport chain complexes (Santos et al. 2013). Similarly, exposure of graphene oxide sheets was found to impair the glutathione redox system of *V. faba* plants in a concentration-dependent manner. At 100, 200, and 1600 mgL^{-1} , graphene oxide induced oxidative stress to plants and increased the level of oxidized glutathione. However, 400 and 800 mgL^{-1} of grapheme oxide reversed the conditions of oxidative stress and increased the reduced glutathione level (Anjum et al. 2013). CeO_2 NP exposure on rice seedlings has documented enhanced ROS production. ROS has induced lipid peroxidation and electrolyte leakage that has further increased H_2O_2 generation. Chlorophyll a photodegradation and enhanced membrane damage was also observed in NP-treated seedlings. Oxidative stress was also evident from lower activity of antioxidant enzymes, namely, GPOX, APOX, and GR enzymes (Rico et al. 2013a).

Exposure of various metal and metal oxide NPs has reduced the dry weight of tomato plants. NP-mediated oxidative stress and membrane damage in tomato plants were responsible for the induced changes (Antisari et al. 2015).

Likewise, CeO_2 NPs also reduced the fresh weight of fruits and nonreducing sugars and antioxidant potential of cucumber plants. CeO_2 NPs have been noticed to alter the starch, nonreducing sugars and antioxidant content of cucumber. CeO_2 NPs enhanced the sucrose content of treated plants. Increased content of starch indicates stress (Zhao et al. 2014).

As mentioned earlier, reduction in plant root length on NPs' exposure demonstrates their phytotoxicity on plants. Free OH group on the surface of alumina NPs has been documented to induce oxidative stress-mediated inhibition of root elongation in maize, *C. sativus*, *G. max*, *B. oleracea*, and *D. carota*. Surface modification of alumina NPs with phenanthrene was observed to reduce the toxic effect on root elongation. Similar reduction in root elongation was obtained in the presence of DMSO, a free hydroxyl radical scavenger (Yang and Watts 2005). Fullerene exposure was found to disrupt the tobacco cell wall. Fullerene increased the glycosyl

residues of the cell wall and also enhanced the ROS content (Liu et al. 2013). Graphene has induced intracellular ROS generation and mitochondrial dysfunctioning in *A. thaliana* cells. Mitochondrial damage and reduced membrane integrity because of increased ROS generation accounted for cell death on graphene exposure. Actually, increased permeability of the outer mitochondrial membrane has enhanced outflux of cytochrome c and other apoptogens from the outer chamber. It has also activated caspases that initiated the process of apoptosis/necrosis (Begum and Fugetsu 2013).

CeO₂ NPs and In₂O₃ NPs induced oxidative stress in *A. thaliana*. However, the less toxicity of In₂O₃ NPs as compared to CeO₂ NPs was due to higher expression of glutathione synthase (GS) genes that are responsible for plant stress management. Hence, plants exposed to In₂O₃ NPs experienced less oxidative stress as compared to CeO₂ NPs (Ma et al. 2013). ZnO NPs induced H₂O₂ production and reduced the level of antioxidant enzymes to induce oxidative stress in *P. sativum* leaves (Mukherjee et al. 2014). CeO₂ and TiO₂ induced ROS generation in *H. vulgare*. ROS further induced chromatin modification in the root and shoot cells (Mattiello et al. 2015). Ag NPs induced oxidative stress to reduce the vegetative growth of *C. abyssinica*. Transgenic *C. abyssinica* plants containing bacterial γ -glutamylcysteine synthase (γ -ECS) were not affected by Ag NP exposure. Actually, transgenic plants contained more glutathione (GSH) content that detoxified the oxidative stress induced by Ag NPs (Ma et al. 2015). The oxidative stress induced by NPs may also be plant growth stage-dependent. CeO₂ NPs induced H₂O₂ generation during the floral growth stage of *Brassica rapa*. H₂O₂ generation was not observed during vegetative growth (Ma et al. 2016). So, increased oxidative stress is a strong reason for toxic effects of NPs on various plants.

NPs, in addition to inducing toxicity, can also enhance the absorption or accumulation of other toxic pollutants present in soil, water, and air. In such a study, MWCNTs induced oxidative stress and nutrient imbalance in *V. faba* seedlings. The oxidative stress was enhanced when seedlings were exposed to MWCNTs in the presence of toxic metal, lead, and cadmium. MWCNTs further enhanced the accumulation of lead and cadmium. As a result, the presence of toxic compounds enhanced the oxidative stress due to MWCNTs as compared to the sole exposure of MWCNTs (Wang et al. 2014). Ag NPs induced growth inhibition and root damage in *T. aestivum* via Ag ions released from NPs. Exposure of NPs altered the level of proteins mainly involved in primary metabolism and abiotic stress management (Vannini et al. 2014). ZnO NPs induced oxidative stress mediated inhibition of *F. esculentum* growth. The activity of ROS enzyme and antioxidant enzymes was increased in NP-treated plants (Lee et al. 2013).

NPs Disturbed the Nutritional Status of Plants

CeO₂ NP exposure was found to reduce the nutritional quality of plants. The accumulation of micro- and macronutrients such as iron, prolamins, glutelin, lauric acid, valeric acid, and starch in grains of NP-treated rice seedlings was significantly

reduced. Reduced antioxidant content of seedlings showed that the NPs were imposing stress. Hence, CeO₂ NPs were creating nutritional deficit in the treated rice seedlings (Rico et al. 2013b).

Likewise, CeO₂ NP exposure considerably reduced the uptake of molybdenum ions by soybean. As a result, the activity of nitrogen fixation enzymes, nitrate reductases, and sulfite oxidases was reduced. So, CeO₂ NPs decreased the nitrogen uptake and nitrogen assimilation of treated plants. Similarly, exposure of ZnO NPs has induced hyperaccumulation of zinc micronutrient in the leaves of soybean. Zn in higher amounts binds with the proteins and displaces other metal ions from the binding sites of proteins. Hence, these NPs have negatively affected the nutritional and commercial value of soybean plants (Peralta-Videa et al. 2014). Ag NPs and FeO NPs have a negative effect on the *T. repens* plant growth through growth inhibition of AMF. FeO NPs did not affect the growth of AMF; instead, FeO NPs were observed to bind glomalin glycoprotein secreted from AMF. Glomalin controls the fluxes of water, gases, and nutrients in soil. So, FeO NP exposure decreases the vegetative growth and antioxidant profile of plants (Feng et al. 2013). Exposure of Ag NPs was observed to delay the vegetative growth of *A. thaliana* by inducing temporary development arrest and reducing the absorption of nitrogenous nutrients by plants (Geisler-Lee et al. 2014).

Citric acid-capped CeO₂ NP treatment was reported to reduce the Ce content and increase the absorption of micronutrients in *R. sativus*. As a result, the citric acid-capped CeO₂ NPs increased the root biomass, while bare CeO₂ NPs reduced root and shoot biomass due to accumulation of more Ce (Trujillo-Reyes et al. 2013).

Si NPs induced toxicity due to adsorption of micro- and macronutrient on the NP surface, thus reducing the nutrition availability for *A. thaliana*. The nutrient adsorption and phytotoxicity of NPs was dependent on pH and surface silanol moieties. No phytotoxicity was observed when the pH of the exposure medium was adjusted to 5.8 and silanol moieties were removed from the Si NP surface (Slomberg and Schoenfish 2012). CeO₂ NPs decreased the nutritional quality of kidney bean (*P. vulgaris*) seeds. The seed produced from CeO₂ NP-treated plants possessed lower amounts of nutrient storage (phaseolin) and carbohydrate metabolism (lectins). Further, the adverse effects on seeds were more pronounced on exposure of CeO₂ NPs through organic matter soil-rich soil than soil containing low organic matter (Majumdar et al. 2015).

Overall Effect on Vegetative Growth Through Other Mechanisms

La₂O₃ NPs were considered toxic to cucumber plants. Plant roots have been known to exude out organic acids in their vicinity. Interaction of acetic acid released from cucumber roots with the La₂O₃ NPs was enhancing its dissolution and its bioavailability and hence induced toxicity to plants (Ma et al. 2011). Likewise, Ag NPs interacted with *P. americana* plant cells to release Ag ions. Ag ion-cell-Ag NP interactions were responsible for the observed phytotoxicity. Size and surface coating were also responsible for the toxicity of Ag NPs. GA-stabilized Ag NPs were

smaller in size and induced more phytotoxicity than PVP-Ag NPs (Yin et al. 2012). Likewise, release of Zn ions from ZnO NPs on interaction with root exudates and the physical interaction of ZnO NPs with plant roots were responsible for the toxicity on rapeseed seedlings (Kouhi et al. 2014). Likewise, Cu NPs and Ag NPs have reduced the biomass of *C. pepo* plants. Ion dissolution from the NPs was considered one but not the sole reason for induced phytotoxicity (Stampoulis et al. 2009).

In contrast, Cu NPs have toxic effect on *P. radiatus* and *T. aestivum* due to nanometer size of Cu. Cupric ion released from Cu NPs was not responsible for toxicity (Lee et al. 2008). Similarly, ZnO NPs inhibited the seed germination as well as root elongation in ryegrass and corn (Lin and Xing 2007; Lin and Xing 2008). Silica, palladium (Pd), Au, and Cu NPs have inhibitory effect on root growth (Shah and Belozerovala 2009). Inhibition of root elongation in rape and wheat on exposure of Yb₂O₃, Gd₂O₃, La₂O₃, and CeO₂ NPs occurred during different stages of seed germination (Ma et al. 2010). While exposure of TiO₂ NPs has induced hindrance in root and shoot growth of wheat seedlings (Mahmoodzadeh et al. 2013). These studies reported that some NP-based mechanism is responsible for the phytotoxicity. Further, the ions released from NPs did not cause any toxicity to the tested plants, suggesting that the nanometric size of particles was responsible for toxicity. Ag NPs induced toxicity by altering the architect of root cell-like vacuolated and collapsed cortical cells and broken epidermis and root cap. Direct interaction of Ag NPs with root cells and interaction of Ag ions generated by Ag NPs with biotic receptors were responsible for the toxicity to roots (Yin et al. 2011).

Further, change in ionic form of cerium CeO₂ NPs was found to induce phytotoxicity to lettuce seeds. It was found that the conversion of Ce (IV) to Ce (III) form was responsible for the decreased growth of lettuce seedlings on exposure. Enhanced lipid peroxidation, altered SOD activity, and membrane damage were also observed in lettuce seedlings on CeO₂ NP exposure (Cui et al. 2014). Exposure of CuO NPs was inducing Cu accumulation in the shoots and roots of *P. vulgaris* plants. Accumulation of excess Cu was responsible for their growth retardation. Supplementation of CuO exposed plants with ZnO induced reduction in the accumulated levels of Cu. ZnO treatment thus counteracted the phytotoxic effect of CuO NPs (Dimkpa et al. 2015).

Au NPs has been observed to induce size-dependent necrosis in tobacco leaves. Interestingly, 3.5 nm NPs entered the plant vasculature through roots. NPs induced toxicity by affecting transport capabilities of vascular tissues. Bigger-sized, 18 nm NPs were not able to enter the plant root. These NPs accumulated outside the roots and hence did not induce toxicity (Sabo-Attwood et al. 2012). Likewise, accumulation of Ag NPs in vascular tissues was reported to inhibit seed germination and seedling growth of rice (Thuesombat et al. 2014). MWCNTs were reported to induce alteration in xylem architecture of red spinach, lettuce, rice, and cucumber plants. Differential xylem architecture was considered responsible for the phytotoxicity (Begum et al. 2012).

Exposure of fullerenes was found to alter the hormonal distribution, cell division, mitochondrial activity, and microtubular organization in *A. thaliana* seedlings (Liu et al. 2010). The finely agglomerated MWCNTs were found toxic to *A.*

thaliana T85 cell lines than loose MWCNT agglomerates. T85 cells have the tendency to clump together, in almost 20 cells, and became a few hundred micrometers in diameter. The cell clumps possessed gorges of a size distinctly smaller for loose MWCNT agglomerates to penetrate. Only finely sized MWCNT agglomerates could enter the plant cell clumps. The plant system, thus, considering the cellular clumps as pathogens, induced hypersensitive response against them (Lin et al. 2009b).

Ag NPs and ZnO NPs induced irregular growth of roots in maize and cabbage due to cell distortion of the meristematic root cells. Ag NPs induced abnormal root growth via narrowing and elongation of root cells. The metaxylem count was also disturbed on Ag NP exposure. While ZnO NPs induced toxicity through tunneling-like effect (Pokhrel and Dubey 2013). CeO₂ NPs have no effect on the yield and vegetative growth of wheat in field experiments. However, CeO₂ NPs decreased the chlorophyll content, altered the root and leaf cell microstructures, and delayed the flowering stage in wheat. Further, the NP-treated plants produced seeds with a smaller starch grain in the endosperm and higher protein content. CeO₂ NPs were observed in seeds of treated plants and, hence, can enter the next trophic level (Du et al. 2015). Ag NPs were more easily internalized by *P. radiatus* and *S. bicolor* in agar medium than from soil. As a result, Ag NPs induced more toxicity in agar medium as compared to soil (Lee et al. 2012).

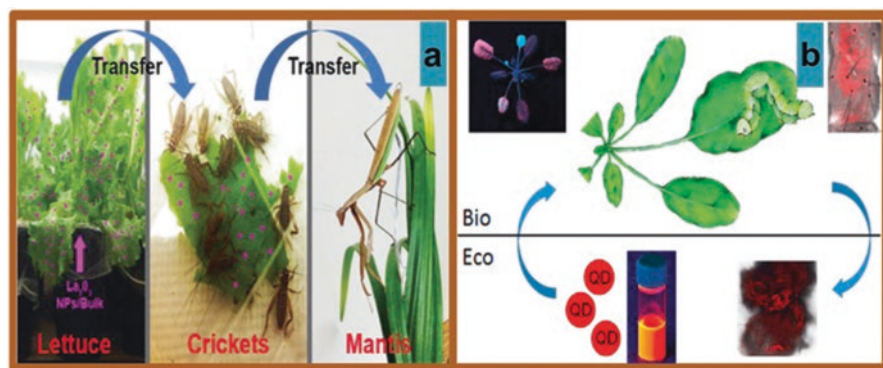


Fig. 10.6 Images showing (a) trophic transfer of La₂O₃ from lettuce plant leaves to mantis. (b). Trophic transfer of QDs from *A. thaliana* to primary consumer *T. ni*. “Reprinted with permission from (Roche et al. 2015; Koo et al. 2015). Copyright (2015) American Chemical Society”

10.4 NPs can Pass Through Tropic Levels: Biotransformation and Biomagnification, a Serious Concern

NPs can transfer from plants to other trophic levels in a food chain or food web (Fig. 10.6). CuO NPs can translocate from root to shoot via xylem, while translocation from shoot to root occurred through phloem. In addition to bioaccumulation and biotransformation, NPs can also be released from roots. The released NPs can interact with microbes in the rhizospheres and affect their growth (Wang et al. 2012b). An inhibitory effect on the growth of *Calotropis gigantea* plants has been documented with exposure to Pb NPs. The leaves of NP-treated plants were reduced in size and number. Further, feeding of painted grasshoppers on Pb NP-treated plant leaves has caused their death. Thus, it suggested the bioaccumulation of lead in plant leaves that induced toxic effects on the plant-dependent grasshoppers (Padmadhas and Ragunathan 2009). So, NPs have the tendency to transfer from one trophic level to another in a food chain. Once CeO₂ NPs entered plants, they can further be transferred to the next trophic level. CeO₂ NPs exposed to kidney bean plants (*P. vulgaris* var. red hawk) get transferred to Mexican bean beetles (*Epilachna varivestis*) when fed on treated plant leaves. Ce content was further transferred to spined soldier bugs (*Podisus maculiventris*) feeding on bean beetles (Majumdar et al. 2016).

Surface charge of cadmium (Cd)-selenium (Se) QDs affected the level of Cd and Se accumulation in the leaves of *A. thaliana*. QDs covered with cationic surfactant polyethylenimine (PEI) were observed to accumulate more Cd and Se than anionic Poly(acrylic acid-ethylene glycol) and neutral poly(maleic anhydride-alt-1-octadecene)-poly(ethylene glycol) over QDs. Cd and Se were further transferred to the next trophic level in *Trichoplusia ni* caterpillars feeding on QD-treated plant leaves (Koo et al. 2015).

On TiO₂ NP exposure to *Aristolochia debilis* plant through roots, NPs were transferred to leaves. The swallowtail butterfly (*Atrophaneura alcinous*) larvae feeding on the leaves not only accumulated TiO₂ NPs but also excreted NPs in the environment through larval excreta (Kubo-Irie et al. 2016). Likewise, Au NPs were transferred from Au NP-contaminated leaves to tobacco hornworm (*Manduca sexta*) caterpillars. The Au NP bioaccumulation was reported in the gut region of caterpillars feeding on Au NP-contaminated *N. tabacum* leaves (Judy et al. 2011; Judy et al. 2012). Pests *Spodoptera litura* F. and *Achaea janata* L. accumulated Ag NPs from PVP-coated AgNP-treated castor plant (*Ricinus communis* L.) leaves. The Ag NPs were accumulated in the gut and induced oxidative stress. NPs were also released through feces in the environment (Yasur and Pathipati 2015). La₂O₃ NPs reduced plant biomass in lettuce (*Lactuca sativa*) plants. Further, the crickets, *Acheta domesticus*, accumulated La₂O₃ NPs by feeding on treated *L. sativa* plants. La₂O₃ NPs were further transferred to the next trophic level to the mantis (*Tenodera aridifolia sinensis*, *Sphodromantis centralis*) feeding on crickets (Roche et al. 2015). These adverse effects can travel up to top consumers of the trophic level. In such a study,

polystyrene NPs were transferred from water to green algae (*Scenedesmus*) to zooplankton (*Daphnia magna*) and finally to top consumer fish (*Carassius carassius*). NPs induced change in the behavior and fat metabolism of fish (Cedervall et al. 2012).

Trophic level transfer of NPs has also been reported in algal-zooplanktons (Gilroy et al. 2014), simplified invertebrates (Holbrook et al. 2008), and terrestrial (Unrine et al. 2012), pond (Marie et al. 2014), and freshwater (Zhu et al. 2010) food chain systems.

10.5 Conclusions

NPs are absorbed by plants and transported to various plant parts in roots and shoots. NPs can have no effect or positive or negative effect on the germination, growth, development, and health of plants. Chemical composition, dose, size, surface covering, and used test plant all affect the response of NPs to plants. Plant response to NP exposure has been explained using various mechanisms. Most common among them is plant growth via enhancing chlorophyll content, photosynthesis, absorption of micro- and macronutrients, and water uptake from soil. However, the negative effect on plant growth and developments are due to induction of oxidative stress, less absorption of nutrients, stress due to toxic metal ions released from NPs, and some unknown NP-based mechanisms. The bioaccumulation and trophic transfer of NPs is also a serious concern and should be thoroughly studied and considered before release of waste containing NPs.

10.6 Future Perspectives

If nanotechnology has to explore its full potential, the toxicity issues associated with NPs has to be managed. As the use of NPs in various consumer products is going to increase in future, there should be proper toxicity evaluation procedures/protocols and guidelines. Plants are the primary land producers, so toxicity evaluation of NPs should be made mandatory. NPs can even pass the trophic barriers, which itself advocates to make nanotoxicity evaluation in plants compulsory.

Proper safety guidelines for using and disposing of NPs are presently lacking. The guidelines, protocols, and reference compound used for toxicity evaluation of macroscopic particles sometimes fail to explain the toxicity of NPs in plants as well as in animals. Further, there are no standard NPs available that can be used as references to evaluate and compare the toxicity of variously synthesized NPs. NPs have unique properties that are not observed in corresponding macroscopic particles. This is due to the reason that the properties of NPs drastically change with even very small change in size, shape, and surface covering. So, microscopic-sized particles of even the same chemical composition cannot be used as reference for NPs. The nanotoxicity evaluation studies are in initial phases. With more research in the plant

nanotoxicity area, policymakers/experts will have to develop some reference NPs and modify the exiting regulatory guidelines and protocols that will enhance the research and application arenas of nanotechnology.

Acknowledgment VK is thankful to LPU, Jalandhar, for providing the necessary research facility. PG would like to acknowledge DST-SERB, GOI, for research grant and DAVU for the research facility.

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Chapter 11

Environmental Impact and Econanotoxicity of Engineered Nanomaterials



Debasree Kundu, Mohd Faheem Khan, Manashjit Gogoi, and Sanjukta Patra

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Springer Nature Switzerland AG 2021

V. Kumar et al. (eds.), *Nanotoxicology and Nanoecotoxicology Vol. 1*,
Environmental Chemistry for a Sustainable World 59,
https://doi.org/10.1007/978-3-030-63241-0_11

Abstract A noteworthy advancement through nanotechnological intervention has been noticed in every sphere of life, including pharmaceutical industry and consumer products. Despite its tremendous benefits, the indiscriminate utilization of nanomaterials in marketed products and their ensuing release into the ecosystems spur serious concern and have potential adverse environmental impacts. However, very little is known on environmental toxicity and risk modeling for nanomaterial emissions to the environment and little or no data exist on reliable quantitative measurements of nanomaterials at actual release concentrations.

In this context, the present work aims to compile and present recent advances, potential hazards and risks to the environment as well as regulatory background of engineered nanomaterials. As many issues regarding the bioavailability, uptake, and the life cycle assessment remain to be explored, we herein highlight and discuss the progress and updates on research of toxicity of engineered nanomaterials used, highlighting the pressing need within the field of econanotoxicity. In addition, grey areas, challenges, and tentative directions for the way forward are suggested.

Keywords Engineered nanomaterials · Bioavailability · Environmental impact · Econanotoxicity · Risk assessment

Abbreviations

AAV	Adeno-associated virus
Ag	Silver
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
Bi ₂ O ₃	Bismuth trioxide
BMF	Biomagnification factor
CdSe	Cadmium selenide
CeO ₂	Cerium dioxide
CNTs	Carbon nanotubes
CrO ₂	Chromium dioxide
EC	Effective concentrations
Eg	Energy gap
EMA	European Medicines Agency
ENPs	Engineered nanoparticles
EPA	Environmental Protection Agency
ET	Evapotranspiration
Fe ²⁺	Ferrous
Fe ³⁺	Ferric
InP	Indium phosphide
LAI	Leaf area indexes
LC	Lethal concentrations

MoO ₃	Molybdenum trioxide
MWNTs	Multiwalled nanotubes
NDA	New drug application
NMs	Nanomaterials
NOECs	No observed effect concentrations
NPs	Nanoparticles
OSHA	Occupational Safety and Health Administration
PEI	Polyethyleneimine
PEN	Project on Emerging Nanotechnologies
PVP	Polyvinylpyrrolidone
QDs	Quantum dots
RCRA	Resource Conservation and Recovery Act of 1976
ROS	Reactive oxygen species
SELs	Size exclusion limits
SPR	Surface plasmon resonance
SWNTs	Single-walled nanotubes
TiO ₂	Titanium dioxide
TSCA	Toxic Substances Control Act
US FDA	United States Food and Drug Administration
ZnO	Zinc oxide
ZnS	Zinc sulfide
ZnSe	Zinc selenide

11.1 Introduction

With the advent of nanotechnology which is of widespread significance, exponential developments have been observed in science and industries like pharmaceuticals, cosmetics, foods, textile, electronics, etc. (Guzmán et al. 2006). Nanoparticles (NPs) or nanomaterials (NMs) are defined as natural or man-made substances that exist in singly or as aggregated/agglomerated form within the range of 1–100 nm (number size distribution in at least one of the dimensions with 50% or more of the particles), along with a volume-specific surface area of at least 60 m² cm⁻³ (EU Commission 2011; Loureiro et al. 2018). More often, nanoparticles are found naturally but their extensive commercial use have put forth the synthetic production of these particles for various tailor-made applications with unique optical, electronic, chemical, biological, and mechanical properties and are termed as engineered nanoparticles (ENPs). Globally, numerous ENP-based products are available for healthcare, energy, and environmental applications (Goswami et al. 2017). Since 2000, the global market value of ENPs has increased from US\$ 125 million to US\$ 7.3–12.7 billion in between 2008–2016. It is slated to reach approximately between US\$ 11.8–16.8 billion by 2022–2025 (Lai et al. 2017; He et al. 2018).

This escalating production and applications of these ENPs results in their exposure in the environmental media and interacts with various trophic levels of the ecosystems. Presently, around 63–91% of ENPs are disposed in landfills while the

remaining are being released into atmosphere (0.1–1.5%), soils (8–28%), and water bodies (0.4–7%) (Keller et al. 2013). Thus, despite multifaceted benefits for commercial purpose, their presence may cause hazardous biological effects in the nature. The unique properties of these nanoparticles leading to detrimental effect in environment mainly comprises of (i) high specific surface area, (ii) sufficient reactive sites on the surface, and (iii) their easy mobility (Wiesner et al. 2006). In this direction, researchers have reported the interactions of nanoparticles with living organisms and little, if any, information is available on the fate and behavior of these nanoparticles within the environment and on human health (Handy et al. 2008). Thus, to narrow the scope of this review, the present chapter aims to emphasize the widespread contamination of the environment due to nanoparticles manufacturing and waste disposal, and highlights the importance of ecotoxicity of engineered nanomaterials to the waste management community.

11.2 Naturally Occurring and Engineered Nanoparticles

With increased anthropogenic activities along with the technological advancements, nanoparticles generate enormous waste materials contaminating the biosphere and pose serious ecological risks. However, nanoparticles still existed and leached into the environment even before the formal emergence of the field of nanotechnology. Naturally occurring nanoparticles are ubiquitous in nature. Several geological processes are known to produce natural nanoparticles such as in the form of combustion by-product, automobile exhaust, aerosols, and volcanoes (Bystrzejewska-Piotrowska et al. 2009). Further, in biological processes, biomolecules like protein, nucleic acids, ATP, membranes, cells, organelles, etc. are directly released into the environment from the organisms, leading to the formation of nanoparticles as a result of degradation of biological matters (Bhatt and Tripathi 2011). However, many of these natural and incidental nanomaterials also have certain distinctive characteristics that cannot be denied from an environmental chemistry perspective (Bernhardt et al. 2010).

Unlike the naturally occurring nanoparticles that are formed heterogeneously and disseminated in the environment, ENPs are mostly homogeneous in terms of size, shape, and structure. The two approaches for the production of ENPs are top-down and bottom-up fabrication method (Bhatt and Tripathi 2011). In the first method, lithographic techniques cut large materials into sizes less than 30 nm. Alternatively, macromaterial are ground in a ball mill for producing NPs having size less than 30 nm (Borm et al. 2006). In contrast, bottom-up synthesis process is a more suitable method to convert extremely small molecules or atoms to nanometer level (Christian et al. 2008). The diameter-tuning of nanoparticles is especially imperative and is regulated with media in which they are synthesized. While temperature and reaction time are important within the realms of wet-phase synthesis protocol, precursor concentration, as well as reaction temperature, controls the diameter of ENPs in gas phase. Moreover, dispersing additives are used to stop

aggregation of the synthesized nanoparticles during mechanical milling; they comprise a film or coat throughout the NPs to prevent aggregation (Borm et al. 2006). However, the unique qualities of the ENPs result in new chemical reactions, thereby making the prediction of its environmental impact and fate more difficult which in turn calls for significant multidisciplinary advances to know about their impacts (Wiesner et al. 2006; Handy et al. 2008).

11.3 Different Classes of Engineered Nanoparticles

As discussed previously, the NPs relevant in the environment can be categorized into natural and engineered nanoparticles. The ENPs are further categorized into various classes, including (i) carbonaceous nanomaterials (fullerene compounds, nanotubes, nanowires, etc.), (ii) metal oxides [bismuth trioxide (Bi_2O_3), chromium dioxide (CrO_2), cerium dioxide (CeO_2), molybdenum trioxide (MoO_3), titanium dioxide (TiO_2), zinc oxide (ZnO)], and binary oxides, (iii) semiconductor materials [quantum dots (QDs)], (iv) zero-valent metals [ferric (Fe^{3+}) or ferrous, dissolution of the metal salt and its reduction to the zero-valent state, etc.], and (v) nanopolymers (dendrimers, liposomes, etc.). Figure 11.1 gives an overview of the nanoparticles and their distribution in the environment.

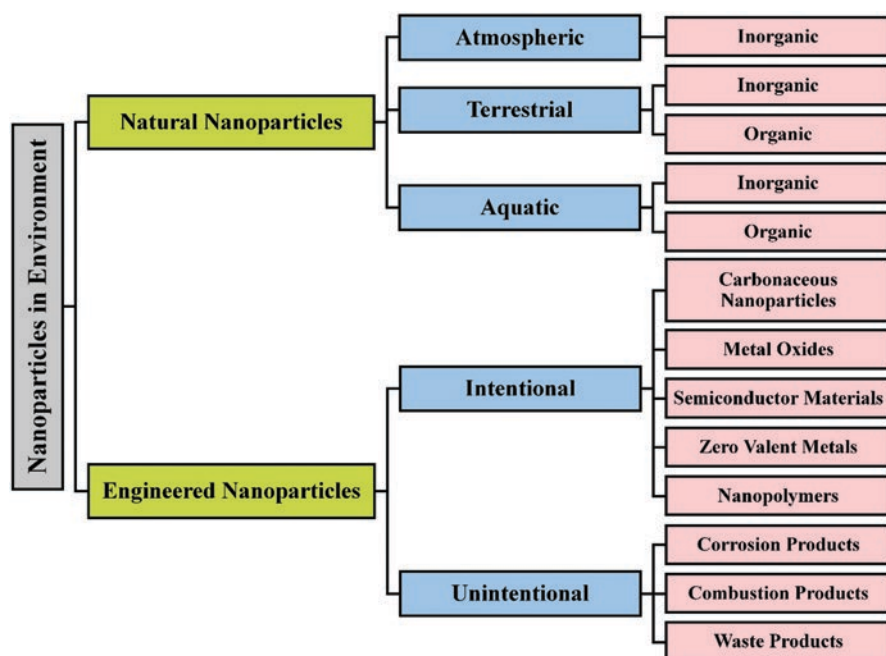


Fig. 11.1 Nanoparticles and their distribution in the environment

Of carbonaceous nanomaterials, the first class of fullerenes (C_{60} -atom hollow sphere) originated in 1985. They are naturally non-ionogenic but gain charge under selective conditions that possess a negative zeta potential and shows optical, elastic, mechanical, and thermal properties (Brant et al. 2005). Further, in 1991, the carbon nanotubes (CNTs), which are the cylindrical fullerene derivative, were synthesized. Sheets of carbon atoms are linked covalently to form one-dimensional hollow cylindrical shape (Smart et al. 2006). CNTs are of two distinct types, namely, single-walled nanotubes (SWNTs) and multiwalled nanotubes (MWNTs). The structure of SWCNTs can be visualized as single-layered graphene sheets that are wrapped up into seamless cylinder. In MWCNTs, two or more concentric layers of graphene sheets with different length and sizes are found (Cao 2004). CNTs and fullerenes find their application in various sectors like medical, plastics, catalysts, fuel electrodes, electrochemical capacitors, wastewater purification system, sensing appliance, etc. (Klaine et al. 2008).

Another type of ENPs comprises of metal-containing materials like metal oxides and binary oxides. The two common methods of their preparation are precipitation with stabilization and flame pyrolysis (Christian et al. 2008). In metal oxides, crystalline TiO_2 is an excellent band-gap semiconductor that has a large energy gap of 3.2 eV (Bellardita et al. 2007; Klaine et al. 2008; Lihitkar et al. 2007; Reijnders 2008). Another example of the same class is ZnO which finds application in cosmetics due to a band-gap energy of 3.36 eV, and high dielectric constant (Singh et al. 2007; Christian et al. 2008).

Quanta dots (QDs) semiconductors with nanocrystalline diameter (2–10 nm) possesses unique magnetic and catalytic properties and constitute the third class of ENPs (Schmid 2004). Examples include core type, core-shell type, or alloyed QDs like chalcogenides of metals (Murray et al. 2001; Logothetidis 2006). They are widely used in experimental medicines, attached to surface ligands or introduced into live organisms for intracellular in vivo analysis, biomedical imaging, targeted therapeutics, etc. (Alivisatos et al. 2005; Roszek et al. 2005; Logothetidis 2006; Klaine et al. 2008).

Nanoscale zero-valent metals that are generally prepared by the reduction of metal salts are also widely used. One such example is the synthesis of zero-valent iron by reducing the ferric (Fe^{3+}) or ferrous (Fe^{2+}) salts with a sodium borohydride ($NaBH_4$). Also, gold and silver NPs are synthesized chemically through metal or metallic salt dissolution in a suitable solvent to reduce them to the zero-valent state (Li et al. 2006). Further, these NPs exhibit unique optical properties called as surface plasmon resonance (SPR) (Noguez 2007).

The last class of ENPs is dendrimers, defined as a complex, highly branched polymers of 1–10 nm diameter. They are asymmetrical and are transformed into globular forms with increase in branching (Caminati et al. 1990). During synthesis of dendrimers, in a process of emulsion polymerization, ammonium per sulfate is used for initiating free radical polymerization. For example, an aqueous emulsion of monomer like styrene or methyl acrylate is prepared using water and sodium dodecyl sulfate or a sulfonate as a surfactant (Shim et al. 2004). Their diverse applications range from biomedicine to surface modification. Some uses of common ENPs are enlisted in Table 11.1.

Table 11.1 The most widely studied ENPs, their properties and applications

Class of ENPs	Diameter ^{a,b} (nm)	Applications
I. Carbonaceous compounds		
1. Fullerenes	0.72	Sorption of organic compounds, removal of organometallic compounds, etc.
2. Carbon nanotubes (CNTs)		For sorption of metals and rare earth metals, water purification systems, electronics, computers, plastics, catalysts, electrodes, supercapacitor, implants, adhesives, composites, sensors, automotive industries, etc.
Single-walled nanotubes (SWNTs)	1–2 (diameter)	
	5–30 μm (length)	
Multiwalled nanotubes (MWNTs)	<8 (OD)	
	2–5 (ID)	
	10–30 μm (length)	
II. Metals and metal oxides		
1. Titanium oxide (TiO ₂)	5	Skin care products, sunscreen lotions, solar cells, paints, bioremediation, etc.
2. Zinc oxide (ZnO)	20	Skin care products
III. Semiconductor devices		
1. Quantum dots (QDs)	1–10	Medical imaging, therapeutics, solar cells, photonics and telecommunications, etc.
IV. Zero-valent metals		
1. Zero-valent iron	20–50	Bioremediation; detoxification of organochlorine pesticides, polychlorinated biphenyls, herbicides, etc.
2. Silver NPs	10	Wound dressings, air filters, toothpastes, baby products, etc.
3. Gold NPs	3–20	Catalyst in flexible conducting inks or films, vector in tumor therapy, etc.
V. Nanopolymers		
1. Dendrimers	1–10	Macrocapsules, nanolatex, colored glasses, chemical sensors, modified electrodes, DNA transfecting agents, drug delivery, tumor treatment
2. Liposome	50–100	Phospholipid vesicles, passive and active delivery of gene, protein, peptide, etc.
3. Polymeric micelles	10–100	Long circulatory, target-specific active and passive drug delivery, etc.
4. Polymeric nanoparticles	10–1000	Controlled and sustained active and passive drug/bioactives delivery

^aThe diameter given is that of a single nanoparticle and often the smallest size commercially available

^bNanoparticles in solution may form aggregates resulting in larger particles

OD represents outside diameter and ID means inside diameter

11.4 Engineered Nanomaterials in Pharmaceuticals: Biological and Environmental Interactions

The introduction and use of nanotechnology in the pharmaceutical industry exhibited remarkable potential and remarkable efforts are in progress worldwide to fulfill the promise of the nanorevolution. Previously, nanotherapies were mainly used as vaccine or cancer therapy, whereas recent trends toward engineering nanomaterials as personalized medicine for the prevention of diseases by employing nanotherapeutics with other advanced nanotechnologies, such as nanobots and nanodevices (Goswami et al. 2017).

The intervention dates back to 1930 when first nanoscale iron colloidal preparation was administered in human. It has been reported that currently there are 43 approved drug formulations commonly referred as nanomedicines, and approximately 789 clinical trials are ongoing pertaining to 25 devices and 122 therapeutics (Weissig et al. 2014; Weissig and Guzman-Villanueva 2015). Milled nanocrystals and liposomes were the first-generation products that used nanomaterials to enhance bioavailability or drug exposure at action sites, respectively for poorly water-soluble drugs. TRICOR[®] is an example that contains active ingredient (Fenofibrate) crystals milled into the nanosize range (Tyner et al. 2015). The first approved new drug application (NDA) was Gris-PEG (griseofulvin ultramicro size, <1000 nm) that targeted treatment of fungal infections. Further, the first US FDA-approved nanotechnology-enabled product was Doxil[®] nanodrug (stealth liposomes encapsulating about 10,000 doxorubicin molecules) that came into being in 1995 for treating AIDS-related Kaposi's sarcoma. Numerous unique features such as (i) increased biodistribution, (ii) enhanced targeting, and (iii) potential of stimuli-sensitive microenvironments payload release facilitated the development of nanotherapeutics of huge antibody–drug conjugates, small-molecule platforms, polymeric nanoparticles, albumin nanoparticles, metal-based nanoformulations, etc. In vaccine therapy, virosomes (e.g., InflexalV[®] and Epaxal[®]), consisting of unilamellar phospholipid membrane nanovesicles integrating virus-derived glycoproteins (100–150 nm) are considered an efficient delivery system. In viral gene therapy, the European Medicines Agency (EMA) in 2012 approved the first product for lipoprotein lipase deficiency that used adeno-associated virus (AAV), allowing stable gene transfer and enduring transgene expression. Various other products, such as aprepitant, fenofibrate, megesterol acetate, and rapamycin are being marketed using the NanoCrystal[®] or the DissoCube[®] technology.

The ever-increasing usage of engineered nanomaterials in pharmaceuticals has provoked scientific community to question their possible negative effect on ecology and animal health. Moreover, the unique properties of engineered nanomaterials make them highly reactive (chemically and biologically), able to interact with the neighboring matters including biological organisms as well as the environmental components that results in toxicity as a result of biological and environmental interactions.

11.5 Physicochemical Properties of Engineered Nanomaterials and Their Toxicity

The indispensable application of the ENPs in different sectors including pharmaceuticals results in their dissemination into the environment. In fact, the very similar properties that direct toward the scientific and technical benefits of nanotechnology also result in exclusive biological effects. Thus, it is imperative to execute physicochemical characterization of engineered nanoparticles like size, shape, structure, surface charge, composition, crystallinity, aggregation, concentration, etc. These properties play significant role in the interaction of the ENPs with the cells thereby leading to toxicity (Fig. 11.2). Hence, the toxicity of the nanomaterials with respect to some of the important physicochemical properties are enlisted here.

11.5.1 Effect of Particle Size

The toxicity of nanomaterials is dependent on its size which in turn is dependent on its capability to move into the biological systems and their modification of structures, thereby interfering with critical biological functions (Lovrić et al. 2005; Aggarwal et al. 2009). Li et al. (2015) suggested that the size of nanoparticles plays a critical role in cellular uptake, efficient processing of particle in the endocytic pathway as well as physiological response of cells to nanoparticles (Li et al. 2015). Various researchers have highlighted the fact that one of the key mechanisms leading to in vivo toxicity of the ENPs is generating oxidative responses due to the formation of free radicals where size has a pivotal role to play. The generated free radicals affect the biological systems mainly through DNA damage, lipid peroxidation, and inflammatory responses. Particles with size below 1 μm enter into cells whereas when the particles are $>1 \mu\text{m}$, the nanoparticles will react with cells through the formation of certain proteins on their surface. Park et al. (2011) compared the various toxicity effects of variable sized silver (Ag) nanoparticles (Park et al. 2011). They inferred that for all toxicity endpoints, 20 nm Ag nanoparticles were more

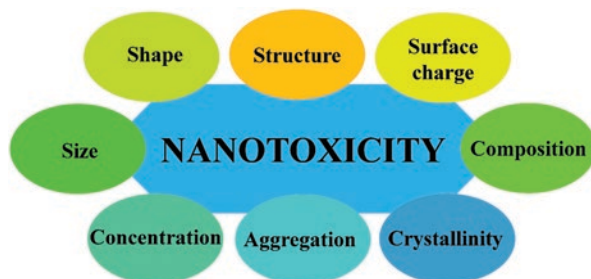


Fig. 11.2 Physicochemical factors of engineered nanomaterials leading to nanotoxicity

toxic than the larger counterparts. However, when compared with Ag ions, Ag particles with size above 20 nm were found to be less toxic than Ag ions. On the contrary to the assumption that small-sized nanoparticles enter the cells more easily causing damages, Yin et al. (2005) witnessed the in vitro effects of particle size on the cytotoxicity of nickel ferrite in Neuro-2A cell line and concluded that the cytotoxicity was independent of the particle size (Yin et al. 2005). Thus, it can be inferred that the mechanism of nanoparticle-mediated toxicity is complicated and size cannot be regarded as the only influential parameter.

11.5.2 Effect of Shape and Structure

Apart from size, toxicity is also dependent on the shape and structure and has been reported for myriads of nanoparticles. Difference in shapes and structure of nanomaterials like planes, spheres, fibers, tubes, polyhydra, etc. often results in alterations in their toxicity. In in vivo, membrane-wrapping processes during endocytosis or phagocytosis are influenced by these ENPs. Endocytosis of spherical nanoparticles is reported to be faster and comparatively less toxic when compared to that of rod or fiber-shaped nanoparticles. Nonspherical nanomaterials more likely flow through capillaries causing other biological consequences (Gatoo et al. 2014). Zhang et al. (2010) conducted a compared toxicity of graphene and carbon nanotubes and found an induction of concentration and shape-dependent cytotoxic effects (Zhang et al. 2010). Moreover, even at low concentrations, graphene induced a stronger metabolic activity emphasizing the effect of shape on cellular toxicity.

11.5.3 Effect of Surface Charge

Surface charge plays a crucial role in toxicity of ENPs as they interact with the biological systems. Surface charge primarily regulates (i) selective adsorption of nanoparticles, (ii) colloidal behavior, (iii) plasma protein binding, (iv) blood–brain barrier integrity, and (v) transmembrane permeability. Mostly, positively charged NPs show enhanced opsonization as well as induce hemolysis and platelet aggregation in comparison to negatively charged and neutral nanoparticles (Goodman et al. 2004). For example, positively charged Si nanoparticles (Si-NP-NH₂) are more cytotoxic in comparison to neutral and negatively charged ones (Bhattacharjee et al. 2010).

11.5.4 Effect of Composition and Crystalline Structure

Toxicity is also influenced by the composition and crystalline structure of nanoparticles. It has been observed that soluble forms of silver and copper nanoparticles triggered toxicity in various tested organisms like zebrafish, daphnids, and algal species, whereas TiO_2 of the same dimensions did not cause any toxicity. Thus, compositions of NPs are integral in determining the toxicities (Griffitt et al. 2008). Regarding crystal structure, it has been observed that rutile TiO_2 nanoparticles induce lipid peroxidation, oxidative DNA damage, and micronuclei formation in the absence of light when compared to the anatase nanoparticles having similar size and chemical composition (Gurr et al. 2005).

11.5.5 Effect of Aggregation and Concentration

Aggregation and concentration can be regarded as the final aspects regarding the toxicity of nanomaterials. Among others, the aggregation of ENPs is mainly dependent on the size, surface charge, and composition. Thus, carbon nanotubes induce cytotoxic effects due to accumulation of aggregates for long span of time (Yang et al. 2008). Further, the pulmonary interstitial fibrosis is enhanced by agglomerated carbon nanotubes than well-dispersed carbon nanotubes (Wick et al. 2007). Regarding the effect of concentration, generally, increase in the nanoparticles concentration leads to decrease in toxicity at higher concentration (Gatoo et al. 2014). Santos et al. (2010) reported that the nontoxic threshold concentration for thermally hydrocarbonized and carbonized porous silicon particles was toxic at 2 mg mL^{-1} , whereas for thermally oxidized porous silicon particles, it was 4 mg mL^{-1} (Santos et al. 2010).

11.6 Ecological Accumulation of Engineered Nanoparticles

There are predominantly three aspects that need to be taken care of while evaluating the impact of engineered nanomaterials in the environmental matrix: (i) their mobility (movement along with transfer) from one place to another or from one recipient to another (for example, from soil to drinking water or food plants), (ii) the possible ecotoxicity to living organisms in aqueous environment, sediments and soils that they likely come into contact, and (iii) to what extent engineered nanomaterials are altered once they are exposed in the environment along with the mechanism behind it. Organisms undergo several routes of exposure to pollutants leading to their uptake. Some of the relevant routes and endpoints are bioavailability, bioconcentration, bioaccumulation, and biomagnification. Table 11.2 enlists some existing and representative biological accumulation studies of synthesized engineered nanomaterials using most commonly used organisms and ecologically relevant contact conditions.

Table 11.2 Ecological accumulation studies of ENPs

Category	Organism	ENP	Salient findings	References
Aquatic invertebrate/ protozoa	Daphnid (<i>Daphnia magna</i>)	C ₆₀ aggregates (10–200 nm)	At 0.2 mg L ⁻¹ nC ₆₀ , estimated BCF (L kg ⁻¹ dry weight) was 15,000 and 437,500 for mother and baby daphnia post 48 h of waterborne exposure	Tao et al. (2009)
		SWCNT coated with phospholipid	At 2.5 ppm SWCNT; Daphnids had uptaken phospholipid-coated SWNT probably through removing/digesting the lipid coating post 96 h of waterborne exposure	Roberts et al. (2007)
		MWCNT oxidized by acid	Post 48 h of waterborne exposure at 0.04–0.4 mg L ⁻¹ CNT, estimated BCF (L kg ⁻¹ dry weight) was 350,000–440,000	Tervonen et al. (2010)
		Degussa P25 TiO ₂ (21 nm, forming ~3500 nm aggregates in exposure solution)	Post 24 h of waterborne exposure, estimated BCF (L kg ⁻¹ dry weight) was 56,563 and 118,063 for 0.1 and 1 mg L ⁻¹ TiO ₂ and 1232 at 1 mg L ⁻¹ TiO ₂ with diet	Zhu et al. (2010)
		Poly(acrylic acid)-octylamine or poly(maleic anhydridealt-1-octadecene)-coated CdSe@ZnS QD modified by PEG (14–43 nm)	With waterborne exposure to 7.7 nM QDs, higher uptake accounted due to PEG modification and were not completely excreted post 48 h depuration	Lewinski et al. (2008)
	Ciliated protozoa (<i>Tetrahymena pyriformis</i>)	Carbonate-coated AgNP (20 nm)	At 0.5 mg L ⁻¹ AgNP, estimated BCF (L kg ⁻¹ dry weight) was 4.6×10^4	Zhao and Wang (2010)
		Citrate-coated Au NP (20 nm)	Post 24 h waterborne exposure at 0.5 mg L ⁻¹ Au NP, it was retained in the gut without any absorption into tissues	Lovern et al. (2008)
		Acid oxidized SWCNT (2–20 × 500 nm)	Post 24 h exposure to 11.9 mg SWCNT L ⁻¹ , it was internalized and hampered ingestion/digestion of diet	Ghafari et al. (2008)
		Citrate-coated CdSe QDs (mean size of 5 nm)	Trophic transfer occurred from preexposed bacteria at 75 mg L ⁻¹ (as Cd) QD to ciliated protozoa with a BMF (dry weight) of 5.4	Werlin et al. (2011)

Aquatic vertebrate	Freshwater snail (<i>Lymnaea stagnalis</i>)	AgNP coated with citrate or humic acid (10–20 nm) ⁶⁵ Cu-labeled CuO NP (spherical shape, 7 nm; rod, 7 × 40 nm)	Ag uptake rates were greater for Ag ion than for AgNP at waterborne exposure of 0.04–9 ppb Ag	Croteau et al. (2011)
	Zebrafish (<i>Danio rerio</i>)	Degussa P25 TiO ₂ (21 nm)	Post 24 h waterborne exposure to 0.2–2000 mg L ⁻¹ Cu, labelled Cu NP distinguished Cu body burden from background versus NP exposure	Misra et al. (2011)
		ZnO (100 nm)	Trophic transfer of TiO ₂ with BMF values (dry weight) <0.024 occurred from preexposed daphnids (0.1–1 mg L ⁻¹ TiO ₂) to zebrafish	Zhu et al. (2010)
		Citrate-coated AuNP (12 and 50 nm)	Post 14 days waterborne exposure, no significant uptake at 0.5–5 mg L ⁻¹ ZnO was reported	Johnston et al. (2010)
Terrestrial invertebrate	Carp (<i>Cyprinus carpio</i>)	Degussa P25 TiO ₂ (21 nm)	Au detected in brain, liver, and muscles in the high exposure concentration (0.1 mg Au per fish per day for 36 days), but not in low exposure level (0.04 mg Au per fish per day for 60 days)	Geffroy et al. (2012)
		AgNP (35 nm)	Post 20 days waterborne exposure to 10 mg L ⁻¹ TiO ₂ , elevated Ti was detected in skin and scale, muscle, gill, and other organs with BCF (L kg ⁻¹ whole body dry weight) of 617	Sun et al. (2009)
		C ₆₀ aggregates	At 0.01–0.1 mg L ⁻¹ AgNP post 21 day waterborne exposure, Ag was detected in liver, bile, intestine, and gills	Gaiser et al. (2012)
	Earthworm (<i>Eisenia foetida</i>)	HCl purified SWCNT and MWCNT	At 0.25 and 100 mg C ₆₀ per kg dry soils, BSAF (kg dry soil per kg dry biomass) was 0.22–0.79 and 0.065–0.13, respectively after 28 days exposure BSAF (kg dry soil per kg dry biomass) was 0.022–0.0061 and 0.014–0.023 for SWCNT and MWCNT, respectively after 28 days exposure	Li et al. (2010) Petersen et al. (2008)

(continued)

Table 11.2 (continued)

Category	Organism	ENP	Salient findings	References
		TiO ₂ (10–20 nm)	Post 7 days exposure at 0.1–5 g kg ⁻¹ dry soil, BSAF was 0.01–0.02 (kg dry soil per kg wet biomass)	Hu et al. (2010)
		ZnO (10–20 nm)	Post 7 days exposure at 0.1–5 g kg ⁻¹ dry soil, BSAF was 0.01–0.37 (kg dry soil per kg wet biomass)	Hu et al. (2010)
		Hydrophilic/amphiphilic coating of AgNP (30–50 nm)	At 10–1000 mg per dry soil of ionic or particulate Ag, ionic Ag was more accumulated than particulate Ag	Shoultis-Wilson et al. (2011)
		Citrate-coated AuNPs (20 and 55 nm)	After 28 days exposure to 5, 20, and 50 mg Au per kg dry soil, particulate AuNP was absorbed into earthworm tissue	Unrine et al. (2010)
	Isopods (<i>Porcellio scaber</i>)	TiO ₂ (<25 nm)	At 5 mg g ⁻¹ food, NP internalization into gut epithelial cells occurred because of cell membrane disruption	Novak et al. (2012)
		ZnO (<100 nm)	Post 28 d exposure to ZnO at 6.2 and 2.5 g ZnO per kg dry leaf, BAF (kg dry leaf per kg dry biomass) was 0.1 and 0.2, respectively	Pipan-Tkalec et al. (2010)

11.6.1 Bioavailability

According to Peijnenburg (2015), bioavailability is the chemical fractions which is accessible or made accessible for uptake causing positive or negative effects in organisms (Peijnenburg et al. 2015). In addition, Ortega-Calvo et al. (2015) defined bioavailability as the component that includes the dissolved fractions of a chemical in soil, whereas bioaccessibility comprises the fraction which may be bioavailable in the long term (Ortega-Calvo et al. 2015). Gaiser et al. (2012) viewed bioavailability in terms of nutritional efficiency, that is, the portion that is taken up, incorporated and utilized for storage and metabolism (Gaiser et al. 2012). The bioactive fraction, in totality, is related to the targeted organelle or particle and the interactions between particles, and thus, to the physiological and biochemical reactions generated and generally termed as biomarkers. It has been observed that engineered nanomaterials in marine ecosystems have a tendency to aggregate more as compared to aqueous freshwater because of surface charge screening in seawater due to high salts, thus, lessening the bioavailability of nanomaterials. Although the bioavailability is decreased, the cited works indicated that engineered nanomaterials are still bioavailable to organisms in marine systems (Table 11.2).

11.6.2 Bioconcentration

Bioconcentration is the procedure through which toxicants are passively absorbed by the living organisms from the environmental matrix exclusively through respiratory and/or dermal surfaces. For quantitatively measuring this process, bioconcentration factor (BCF) is conventionally calculated which is expressed as the ratio of the particle concentration in an organism to that in exposure medium (usually water or medium). BCF, expressed in terms of $L\ kg^{-1}$, are usually expressed as chemical mass per L and chemical mass per kg biomass, respectively. BCF is measured at its steady state and is a net effect of uptake and elimination processes, taking care of metabolic transformation, fecal egestion, gill elimination, and growth dilution. The approximation of BCF can invite a few ambiguities as literature reports are either merely abstractive or it is difficult converting to BCF used for assessment. For example, the mean log BCF values for daphnids in case of many ENPs are quite broad, and vary from 3.16 to 5.64. On the other hand, the engineered nanomaterials mean log BCF values in fish varies from 1.27–2.87, which are 1–2-folds lesser than those of daphnids (Hou et al. 2013). However, bioconcentration can only be determined in controlled environmental settings.

11.6.3 Bioaccumulation

Bioaccumulation occurs if exposure takes place through contaminated food along with ambient sources and bioaccumulation endpoint (bioaccumulation factor, BAF) is defined as the ratio of the concentration of the substance or chemical in an organism (specific genus) (chemical mass per kg biomass) to the exposure concentration in water (chemical mass per L) (Hou et al. 2013). However, in case of exposure to soil or a benthic environment, the bioaccumulation endpoint is typically characterized by the ratio of chemical concentration in an organism to that in the sediment and termed as biota-sediment accumulation factor (BSAF). As per the USEPA Toxic Substances Control Act (TSCA), bioaccumulative substances have log BCF values in the range 3–3.7 and those with log BCF values ≥ 3.7 are considered very bioaccumulative substances. It has been shown that the bioaccumulation potential of nanoparticles to fish through oral route or food exposure is relatively low. In case of earthworms, several reports have revealed that the bioaccumulation potential of metal oxide or metallic nanoparticles.

11.6.4 Biomagnification

Biomagnification is the accumulation of a toxicant or chemical or pollutant by an organism due to water and food intake and it results in a concentration higher than that would have achieved from water contact alone and thus higher than expected from equilibrium (Hou et al. 2013). The biomagnification end point, the biomagnification factor (BMF), is the extent to which the concentration increases from one trophic level to next higher level. More precisely, BMFs are expressed as the ratio of the fugacity of a chemical entity in the predator to that in the prey, rather than as an expression of concentrations as discussed just above. In general, a BMF >1 signifies that biomagnification exists in a given food web. The comparatively greater bioaccumulation and partial depuration of engineered nanomaterials in lower trophic level organisms like daphnid results into the chance for trophic transfer and biomagnification through the food chain. Werlin et al. (2011) reported that CdSe QD titer in ciliated protozoa (*Tetrahymena thermophila*) is ~5 times higher than that in the bacteria (*Pseudomonas aeruginosa*), demonstrating that biomagnification occurs (Werlin et al. 2011). In contrast, due to the lack of QD internalization into bacterial cells, Holbrook et al. (2008) failed to observe trophic transfer from bacteria (*Escherichia coli*) to ciliates (*Tetrahymena thermophila*)-rotifers (*Brachionus calyciflorus*) (Holbrook et al. 2008). The difference would imply that uptake of QDs by bacteria is dependent on microbial isolates and/or QD exterior functionalization. In the absence of bacteria, QDs could be uptaken by ciliates and trophic transferred to the predator, rotifers. However, the body burden in rotifers is less than that in ciliates (BMF = 0.29–0.62), implying no biomagnification. Trophic transfer has also been observed in many high trophic level aquatic food webs, including QDs and Ag NPs transfer from algae to daphnia, QDs or nTiO₂ transfer from daphnia to fish, clamworm to juvenile turbot.

11.7 Toxicity and Environmental Impact of Nanoparticles

Eventually, most of the ENPs are considered to be xenobiotic in nature and their potential release and fate pattern remain poorly understood (Mraz 2005; Oberdörster et al. 2005). Toxic NPs generate reactive oxygen species (ROS) that causes damages to membrane stabilization, protein damage and oxidation, nucleic acids degradation, release of harmful and toxic components, etc. (Klaine et al. 2008). Since ENPs are extensively used in biological applications, variable doses of ENPs should be administered *in vivo* to evaluate the ecotoxicological aspects of ENPs (Kunzmann et al. 2011).

Figure 11.3 explains the mechanisms of toxicity exerted by ENPs in living organizations. Once ENPs enter the living organism through endocytic pathways via motor proteins and cytoskeletal structures, they get transferred to the endolysosomal network within vesicles. Thereafter, the ENPs traverse the cytoplasm gain to access the nucleus causing cytotoxicity in the host organism (Shang et al. 2014). Moreover,

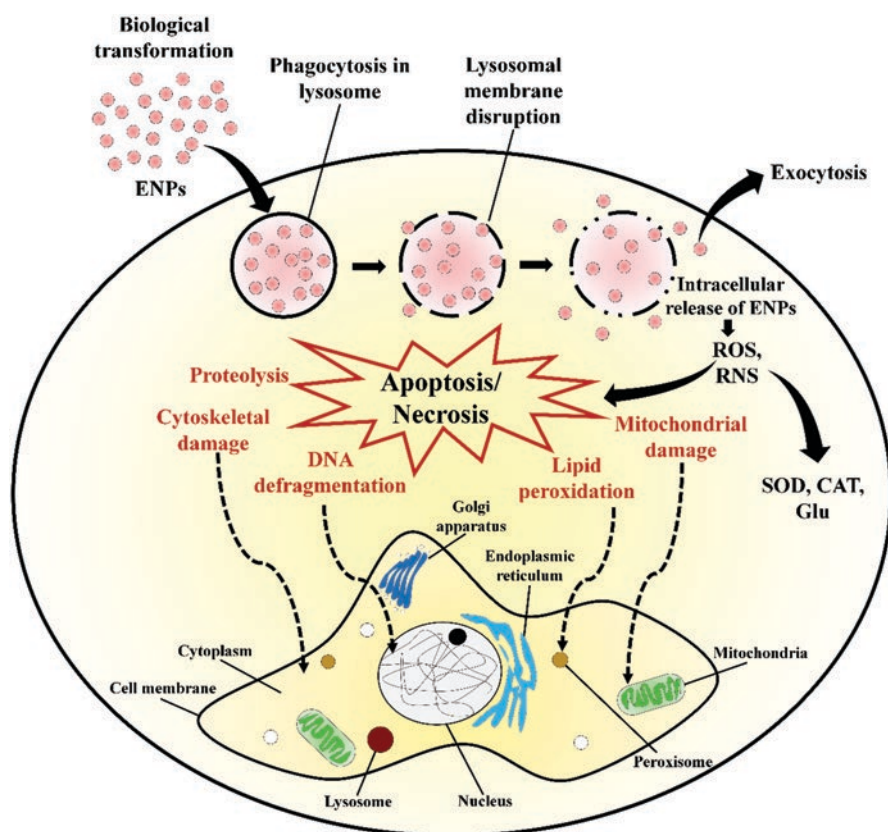


Fig. 11.3 Schematic representation of ENPs generated cytotoxicity

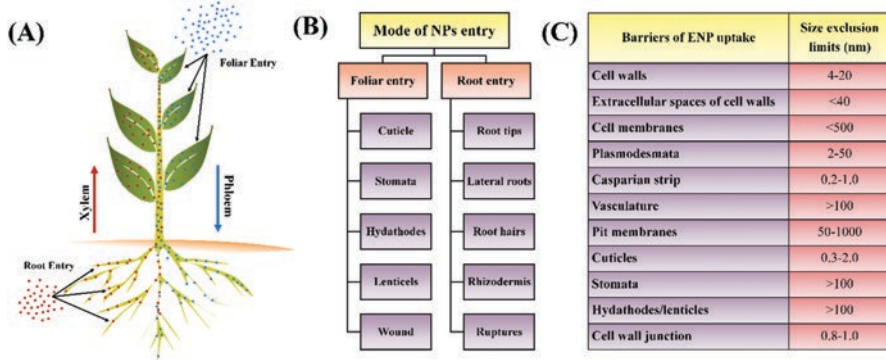


Fig. 11.4 (A) and (B), Mode of entry of ENPs in plants; (C) size exclusion limits of barriers for the uptake and transport of ENPs in plants

ENPs may interact with membrane-bound cellular receptors like growth factor receptors and integrins to induce proliferation, differentiation, and migration.

In microbes, ENPs are accumulated either in the cells or adhere near the cell wall as electron dense structures (Feng et al. 2000). Accumulation of inorganic nanoparticles mostly occurs in the cytoplasm. This leads to the damage of bacterial membrane and create the access of NPs easy leading to the modification in cell by intracellular potassium leakage (Navarro et al. 2008). It must also be noted that surface coatings comprising of simple or complex organic moieties can act as carbon source for bacteria.

Further, plants also regularly encounter nanoparticles in environmental matrix. While atmospheric nanoparticles are shown to be adhered to leaves and other aerial parts of plants, roots encounter in proximity with aquatic or soil matrix nanoparticles. Thus, the entry points of ENPs in plant tissues are either through the underground roots or the aerial parts (e.g., cuticles, trichomes, stomata, stigma, and hydathodes), together with wounds and root joints (Fig. 11.4A, B). ENPs must navigate a sequence of biotic and abiotic barriers for uptake and translocation (Fig. 11.4C). The ENPs are internalization into the cells from the cell wall and occur through endocytosis (Corredor et al. 2009). Successive symplastic transfer afterward is dependent upon the size control limits of the plasmodesmata (Šamaj et al. 2004). Many literature reports suggest that ENPs have been found both in the apoplast and symplast; however, it remains to be established which route is more dominant.

Over the past decade, many experiments have been performed on the short-term acute toxic effects of ENPs. However, elucidation on chronic endpoints is relatively new area of research and it received impetus only after the first forum convened in Stockholm in 2007 (Kostarelos et al. 2007). Reports from the US EPA has revealed that titanium dioxide nanoparticles used in cosmetics have the potential to create brain damage in mice (Long et al. 2006). Nanosized titanium dioxide generates reactive oxygen species in brain microglia and affects neurons in vitro (Long et al.

2006). Most metal oxide nanoparticles show genotoxic and cytotoxic properties on fish cells (Handy et al. 2008; Vevers and Jha 2008). In the presence of magnetic nanoparticles of <10 nm dimension, neuronal cells enter a latent state and stop to react to chemical signals (Johnson 2007). Fullerenes are also reported to kill liver, skin and brain cells in vitro (Lewinski et al. 2008). Those nanoparticles which have been degraded in the cellular *milieu* could build up intracellularly, leading to either gene modification(s) or destruction of organelle integrity. Carbon-, metal-, and semiconductor-based nanoparticles, at high doses, exert cellular toxicity effects in a dose- and time-dependent manner. In case of reproductive system, literature cited works suggest that nanoparticles accumulate in the testes by traversing the blood–testes barrier and exert damage on sperm cells (McAuliffe and Perry 2007).

11.8 Risk Assessment of Engineered Nanoparticles

Repeated release as well as contact of ENPs with many elements of environment and trophic levels and likely hazards call for the strategy development or set patterns to test the probable risks of engineered nanomaterials. The toxicity of engineered nanomaterials is dependent upon their basic physicochemical properties and added functional chemico-biological features. Thus, these basic properties need to be evaluated while investing their probable ecological toxicity that becomes difficult because (i) their actual concentration in environment is much less than the measurable limits for most experimental tests and (ii) in addition to intentional ENPs, environment also consists of naturally produced NPs (Lead and Wilkinson 2006). Therefore, development/updating of currently available system to attain an improved screening potential and high selective recognition are the prerequisite. The first step toward this is pre-fractionation, that is, reduction of the mixture of particles in the real samples using stirring, centrifugation, or filtration. Size fractionation can be achieved through membranes (ultrafiltration, nanofiltration, and dialysis) as well as chromatography (Hassellöv et al. 2008). After that the size of ENPs can be examined through several instrumentations, light scattering is a frequently used method. Post analysis and characterization of ENPs, both the short- and long-term effects of ENPs on living organisms are tested. Establishment of a dose–response relationship by subjecting the organism to varying concentrations of NPs is a common pattern in almost all nanotoxicity-related studies (Navarro et al. 2008). The environmental hazards associated with chemical substances are assessed through standard ecotoxicity tests that focuses on the target/nontarget test species, endpoints protocols and measurement. The standard endpoints that are calculated [for example, lethal concentrations (LC), effective concentrations (EC) or no observed effect concentrations (NOECs)] are usually for higher organisms. In case of microbes and algae, the endpoint is population growth because of their fast growth (Crane and Scott 2012).

11.9 Nanowaste: Guidelines/Regulatory Measures

While the exponential growth of nanotechnology offers many benefits, they also contribute in generating wastes. Many of the nanomaterials-based manufacturing and products are discharged into the environment as a result of their disposal in waste streams (Moore 2007; Powell et al. 2008). Currently, industrial data on handling of discarded nanomaterials and their end-of-life scenarios remain elusive. At present, there is no centralized policy explicitly to tackle the ecotoxicity and safety inference of nanotechnology. There are no national or global safety guidelines or regulatory measures on manufacturing and characterization for nanomaterials at workplace. At the moment, regulatory government bodies in the USA (i.e., EPA, FDA, NIOSH) and in European Union (i.e., OECD, ECHA) have drafted strong technical guidelines and legislations to control the potential risks of ENPs. Considering the lack of the current risk assessment model and regulatory frameworks, the Woodrow Wilson International Center Project on Emerging Nanotechnologies (PEN) have highlighted the end-of-life directive of nanotechnologies (Breggin and Pendergrass 2007). However, much like usual chemical substances, research, and development with ENPs must be accomplished with great safety and responsibility. All federal, state, and local requirements must be dealt with while handling, transporting, storing, using or disposing chemicals, including nanomaterials.

The Occupational Safety and Health Administration (OSHA) stress on employers to sustain a secure and healthy working environment, “free from recognized hazards likely to cause death or serious physical harm”. As per OSHA guidelines, training and orientation programs on material safety data sheets and labeling and signage must be performed to educate the laboratory personnel so as to make them aware of the risks associated with workplace hazards. The transportation, treatment, disposal, and cleanup of hazardous waste come under the purview of The Resource Conservation and Recovery Act of 1976 (RCRA). Nanomaterials that have potential to be treated as a “hazardous waste” in RCRA are subject to this rule. Nanomaterials that are “chemical substances” under the Toxic Substances Control Act (TSCA) and which are not on the TSCA Inventory must be reported to the US Environmental Protection Agency (EPA). The usual practice is that a chemical substance that is not on the TSCA Inventory of Chemical Substances must be manufactured or imported with a prior “Premanufacture Notice” submitted to the EPA.

In case of all commercially available new pesticide products, the US EPA approval is necessary as per the ‘Federal Insecticide, Fungicide, and Rodenticide Act’ before registration along with subsequent evaluation, product composition and characterization, proper labeling mentioning proposed use of the material and data of extensive health and safety testing need to be submitted to US EPA. Furthermore, the US Food and Drug Administration also presently regulate an extensive array of nanotechnology or nanomaterials-enabled products (e.g., a nanomaterial for biomedical use).

11.10 Concluding Remarks, Challenges, and Perspectives

As the potentials and possibility of nanomaterials is well established, green and sustainable growth of nanotechnology is particularly imperative keeping environmental concern in mind. Environmental legislations must be promoted to develop ENPs with innovative parameters such as minimal mobility in environmental media and little or no toxicological effects for humans and ecology. The amount of introduction of nanoparticle in the ecological media is mounting speedily due to the numerous alternative green methods available today in both academia and industry. To date, enumeration of analytical environmental concentrations (hazard and exposure) of many popular nanoparticles is still not available. However, release and monitoring of ENPs/ENMs are required to be computed based on risk assessment and life cycle design concept.

Furthermore, to comprehend the long-lasting effect of ENPs/ENMs on the human health and ecology, extensive ecotoxicological data regarding their bioaccumulation and trophic transfer are required.

While it is widely accepted that many cited works have been carried out over the past decade, it goes without saying that the potential negative effects of engineered nanoparticles have been neglected. Here, we review the major observations emanating from recent works.

- There is dearth of evidence on the transformation of engineered nanoparticles.
 - (a) For instance, how transformations take place or expected at various conditions such as types of electrolytes used and their concentrations; pH of the preparing solution; nanoparticles' particle size and effect of coating, if any; interactions with the environmental media and different physicochemical conditions, etc.? What are the transformation pathways of the nanoparticles? How does bioactivity and biotransformation or modification affected by media composition and trophic interaction?
 - (b) How many potential stable species of transformed/aged nanoparticles exist in natural media and how do they interact with biota?
- In-depth evidence from in vivo studies is required to truly reflect on fate, behavior, and transport of the engineered nanoparticles as the in vitro ecological studies do not necessarily mirror the factual effects of engineered nanoparticles in natural environmental media.
- Although a plethora of literature data are available on greener synthesis of nanoparticles, there is lack of approach toward cost-effective quality by design products, a thorough appreciative understanding and production of safer by design (ecosafe) products.
- Long-term experiments along with life cycle analysis to squarely reflect the release and exposure conditions at all ecosystem level are crucial to minimize the possible ecotoxicity of nanoparticles in different species.

- The effects of forms (single or clustered, pristine or transformed), aging, transformation (both chemical and biological), and elemental compositional analysis and speciation on the inventory analysis warrant immediate attention.
- The information about how the large-scale productions of nanoparticles affect the long-term impact in an ever changing environment is essential.

In a nutshell, addressing these research gaps and agglomeration on a common research platform is required to extend a rational framework for safeguarding the ecology that will result in a greener and safer earth around.

Acknowledgments Debasree Kundu acknowledges Science and Engineering Research Board (SERB), New Delhi for providing National Postdoctoral Fellowship (File No. PDF/2015/000382). Mohd Faheem Khan is thankful to Indian Institute of Technology Guwahati for providing research fellowship to pursue doctoral studies.

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